Der Apoptoseinhibitor Survivin als molekulare Zielstruktur einer Modulation der intrinsischen Strahlenresistenz von Tumorzellen

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Dipl.- Biol. Sebastian Reichert

aus Saarlouis

Berichterstatter: Prof. Dr. Markus Löbrich

Mitberichterstatter: Prof. Dr. Bodo Laube

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Inhaltsverzeichnis

1. Einleitung

Ein wesentliches klinisches Problem der Tumortherapie stellt das individuell differenzierte Ansprechen der Patienten auf die Behandlung dar. So zeigt beispielsweise das Monitoring der Therapieantwort auf eine Strahlen- oder kombinierte Radiochemotherapie durch bildgebende Verfahren (PET- oder MRT-Technologie), sowie durch histopathologische Regressionsbeurteilung, dass die Tumorantwort im Einzelfall trotz identischer Therapie, gleicher Histologie und vergleichbarer Tumorstadien erheblichen Schwankungen unterliegt [95]. Diese reichen von einer klinisch und pathologisch kompletten Remission ohne Nachweis vitaler Tumorzellen bis hin zu einer Tumorprogression. Für dieses unterschiedliche Ansprechen bei meist homogener Behandlung werden unter anderem zelluläre Resistenzmechanismen bei individuell unterschiedlicher Genexpression verantwortlich gemacht [43]. Daher ist es von klinisch herausragender Bedeutung, molekulare Faktoren zu definieren, die das Therapieansprechen vorhersagen und so eine Individualisierung der Therapie ermöglichen könnten. Diese molekularen Zielstrukturen sind zudem potentielle Angriffspunkte für eine klinische Intervention im Sinne einer zielgerichteten („*targeted*“) Tumortherapie.

Einen in diesem Zusammenhang interessanten Faktor stellt das im Jahre 1997 von Ambrosini erstmals als Mitglied der „*Inhibitor of Apoptosis Protein*“ (IAP)-Familie beschriebene Survivin dar [9]. Im Gegensatz zu anderen Mitgliedern der Familie wird Survivin im Tumorgewebe stark überexprimiert und spielt eine essentielle Rolle in der Tumorzellresistenz gegenüber Chemotherapie und ionisierender Strahlung [55,69,97]. Für eine Vielzahl von Neoplasien, wie Tumoren der Lunge, der Blase, des Rektums und der Kopf- und Halsregion wurde eine erhöhte Expression von Survivin als prognostischer Marker für Tumorprogression und verkürzte Überlebenszeiten beschrieben [36,88,99,126]. Darüber hinaus wurde Survivin als Chemo- und Radioresistenzfaktor erkannt und befindet sich aktuell als Zielstruktur einer molekular zielgerichteten Tumortherapie in der klinischen Prüfung [44,53,96,103]. Da die zelluläre Funktionalität und Möglichkeiten zur Verbesserung der klinischen Anwendbarkeit von Survivin-Antagonisten Hauptgegenstand der Arbeit sind, werden im Folgenden die Struktur, die Rolle in der Bestrahlungsreaktion und die klinische Relevanz von Survivin detailliert dargestellt.

1.1 Struktur und Funktion von Survivin

Der Genlokus für humanes Survivin (BIRC5) liegt auf Chromosom 17q25 und kodiert für ein 16,5 kDa großes Protein mit 142 Aminosäuren, welches strukturell durch eine, für die IAP Familie charakteristische *Baculovirus-IAP-Repeat* Domäne, eine Cystein/Histidin Zink-Finger-Domäne und eine α-helikale „*Coiled–Coil-Domain*“ gekennzeichnet ist [9,67,122]. Die transkriptionelle Kontrolle der Expression erfolgt in nicht transformierten Zellen zellzyklusabhängig über sogenannte-Boxes *Cell-Cycle-Dependent Element/Cell-Cycle Gene Homology Region* (CDE/CHR), die dem Survivinpromotor vorgelagert sind. Zusätzlich wird das Protein in der G2/M-Phase durch den $p34^{cdc2}$/Cyclin-B-Kinasekomplex phosphoryliert und dadurch stabilisiert [70,72,122]. Wesentlich für eine negative Regulation der Survivin-Expression erscheinen auch das Wildtyp Tumorsuppressorprotein p53 und das Wildtyp *Adenomatous Polyposis Coli* (APC) Protein [21,51,133,134]. Da diese regulatorischen Proteine jedoch mit einer hohen Inzidenz in Tumorzellen mutiert sind, stellt dies einen Mechanismus der Überexpression von Survivin in Tumorzellen dar. Als weitere Mechanismen, die zu einer gesteigerten Expression von Survivin in transformierten Zellen beitragen, wurden eine Amplifikation des Survivin Genlokus, eine Demethylierung des Promotors oder eine erhöhte Promotoraktivität beschrieben [48,52,72]. Die Letztgenannte kann durch onkogene Faktoren wie c-H-Ras, c-Myc oder auch *wingless-type MMTV integration site family* (WNT)*/ß-Catenin/Transcription Factor 4* (TCF4) induziert werden [37,109]. Darüber hinaus tragen Transkriptionsfaktoren wie *Signal Transduction and Activator of Transcription 3* (STAT3) und *Nuclear Factor-κB* (NF-κB) zu einer transkriptionellen Regulation des Proteins bei [45,46,58]. Survivin wird zudem in vielfältigen Mechanismen posttranslational modifiziert. Zu diesen zählen Ubi- und Deubiquitinierung, Acetylierung und Phosphorylierungen [123,137]. Bisher bekannte Phosphorylierungsstellen, welche die Stabilität und subzelluläre Lokalisation vermitteln, umfassen die Aminosäuren Serin 20 (*Protein Kinase A* und *Polo-like Kinase 1*), Threonin 34 (*Cyclin Dependent Kinase 1*) und Threonin 117 (*Aurora Kinase B*). Darüber hinaus wird das Protein durch Bindung an das Chaperon *Heat shock protein 90* (Hsp90) stabilisiert und vor dem Abbau durch den Ubiquitin-28S Proteasom-Reaktionsweg geschützt [25,30,38,87,127].

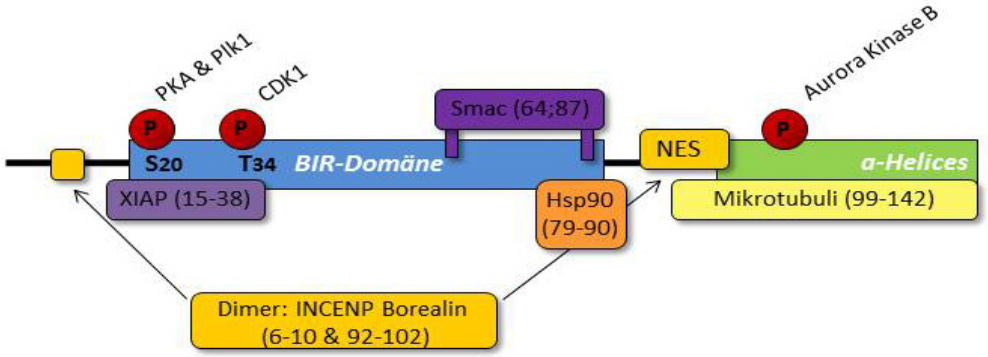

***Abbildung 1**: Struktur und funktionelle Einheiten von Survivin (nach [98]).*
Dargestellt sind die funktionellen Domänen (Baculovirus Inhibitor of Apoptosis Repeat (BIR)-Domäne und C-terminale α-helikale Domäne), die Phosphorylierungsstellen Serin (S)20 (Proteinkinase A (PKA) und Polo-like Kinase 1 (Plk1)), Threonin (T)34 (Cyclin-Dependent Kinase 1 (CDK1)) und T117 (Aurora Kinase B) sowie die Bindungsstellen für Interaktionspartner wie X-linked Inhibitor of Apoptosis Protein (XIAP), Second mitochondria-derived activator of caspases (Smac), Heat shock protein 90 (Hsp90), Mikrotubuli und das Dimer aus Inner Centromere Protein (INCENP) und Borealin. Eine nukleäre Export Sequenz (NES) bewirkt einen aktiven Transport des Proteins aus dem Zellkern.

Funktionell stellt Survivin ein zentrales (nodales) Protein mit multiplen Funktionen in einer Vielzahl zellulärer Netzwerke der Tumorzelle, einschließlich der Zellteilung, Apoptose und der zellulären Reaktion auf ungünstige Umgebungsbedingungen (Stressantwort) dar [5-7].
In der Prometaphase und Metaphase der Mitose assoziiert Survivin mit der *Aurora Kinase B*, Borealin und dem *Inner Centromere Protein* (INCENP) und stellt eine wesentliche Komponente des *Chromosomal Passenger Complex* (CPC) dar, der die Chromosomen-segregation mit der Zytokinese verbindet [40,49]. Zudem bindet Survivin an die Mikrotubuli der mitotischen Spindel, an Zentromere und an Kinetochore und ermöglicht so eine koordinierte Zellteilung [100,128]. Im Gegensatz dazu führt eine Hemmung der Survivin-Expression zu einer unvollständigen Zytokinese mit dem Auftreten von multipolaren Spindeln und Hyperpolyploidie [23,71].
Obwohl die molekularen Mechanismen der Apoptoseregulation durch Survivin noch nicht vollständig geklärt sind, stellt die Inhibition der Effektor-Caspasen 3, 7 und 9 - und damit der gemeinsamen enzymatischen Endstrecke des apoptotischen Programms - eine wesentliche Komponente der Wirkung dar. Aktuelle Vorstellungen besagen jedoch, dass Survivin, ähnlich wie andere Mitglieder der IAP-Familie (mit Ausnahme von *X-linked Inhibitor of Apoptosis Protein* (XIAP)), nicht in der Lage ist Caspasen direkt zu hemmen, sondern für diese Aktivität Bindungspartner benötigt. So ist eine Interaktion mit *Hepatitis B X-Interacting Protein*

(HBXIP) und mit XIAP beschrieben, die eine Aktivierung der Pro-Caspase 9 und den proteasomalen Abbau von XIAP verhindert [32,75]. Weitere Modelle zur Apoptosehemmung durch Survivin beinhalten die Bindung von Survivin an das mitochondriale Protein *Second mitochondria-derived activator of caspases* (Smac/Diablo), welches die inhibitorische Wirkung anderer Mitglieder der IAP-Familie auf Caspasen zu antagonisieren vermag [31].
Neben der ausgeprägten Fähigkeit zur Wechselwirkung mit einer Vielzahl von Bindungspartnern, stellt der Nachweis von Survivin in unterschiedlichen Zellkompartimenten ein weiteres Charakteristikum des Proteins dar. So kann Survivin im Zellkern, im Zytoplasma, im Mitochondrium und, wie aktuell gezeigt, auch im extrazellulären Raum nachgewiesen werden [29,39,59,112]. Obwohl das niedrige Molekulargewicht von 16,5 kDa theoretisch eine passive Diffusion in diese Kompartimente ermöglichen könnte, sind für den Transport in das Mitochondrium (mittels Chaperon Hsp90) und für den Export aus dem Zellkern aktive Transport („*Shuttle*“) Mechanismen bekannt [28,56]. Wesentlich in diesem Zusammenhang erscheint eine nukleäre Export Sequenz (NES), die einen aktiven *Chromosome Region Maintenance Protein 1 Homolog* (Crm1) vermittelten Transport aus dem Zellkern ermöglicht [24,62,113]. Die differentielle Lokalisation von Survivin spiegelt unterschiedliche Funktionen des Proteins wider. So wird zytoplasmatisches Survivin als essentiell für die Regulation der Apoptose angesehen, während nukleäres Survivin überwiegend zur Mitoseregulation beiträgt [112].

1.2 Survivin als Strahlenresistenzfaktor

Aufgrund der zentralen Funktion in der Regulation der Apoptose und Aufrechterhaltung der Zellviabilität wurde für Survivin schon früh eine Funktionalität in der zellulären Strahlenreaktion postuliert [78]. Tatsächlich konnten Asanuma und Mitarbeiter im Jahre 2000 erstmals in Pankreaskarzinomzellen eine inverse Korrelation der Survivin mRNA-Expression mit der Strahlensensibilität und einen Anstieg der Expression nach subletalen Strahlendosen zeigen [11]. Survivin kann somit als induzierbarer Radioresistenzfaktor angesehen werden. Dieser Zusammenhang wurde auch in Untersuchungen an kolorektalen Zelllinien bestätigt, die eine enge Korrelation zwischen dem Ausmaß der Survivin-Expression, der Apoptosefähigkeit und der Strahlensensibilität nachweisen [94]. Nachfolgende Studien zeigten übereinstimmend, dass eine funktionelle, *small interfering* (si)RNA-, Antisense-

Oligonukleotid- oder durch Einsatz von dominant-negativen Mutanten vermittelte Hemmung von Survivin zu einer signifikanten Steigerung der Strahlensensibilität führt, die auch in Xenograft Tiermodellen bestätigt werden konnte [16,55,96,97,117,130]. Die strahlensensibilisierende Wirkung einer Survivin Hemmung beruht dabei nicht nur auf Caspase-abhängigen, sondern, wie mehrere Arbeitsgruppen zeigen konnten, auch auf Caspase-unabhängigen Mechanismen [17,20,53].

So wurde gezeigt, dass die Suppression von Survivin in Tumorzellen zu einer gesteigerten Anzahl an Zellen in der G2/M-Phase und damit in der Phase des Zellzyklus mit der höchsten Strahlensensibilität führt [26,84]. Chakravarti und Mitarbeiter waren zudem die ersten, die einen möglichen Einfluss von Survivin auf die Reparatur von strahleninduzierten Doppelstrangbrüchen postulierten [20]. Nach adenoviraler Transfektion einer dominant-negativen Survivin T34A Mutante konnte die Gruppe mittels Einzelzell-Gelelektrophorese (*Comet-Assay*) nach Survivin-Attenuation eine beeinträchtigte DNA-Reparatur in Glioblastomzellen nachweisen. Dieser Zusammenhang wurde nachfolgend durch Messung der Kinetik einer γ-H2AX Foci-Bildung bestätigt [20]. Die Histonvariante H2AX wird rasch nach Induktion von DNA-Schäden an Serin 139 phosphoryliert und kann mittels Nachweis mit einem spezifischen Antikörper als Marker für das Auftreten und die Reparatur von DNA-Doppelstrangbrüchen (DNA-DSB) verwendet werden [53,97].

Aufgrund der hohen Relevanz der DNA-DSB für die genetische Stabilität existieren eine Reihe von komplexen DNA-Reparaturmechanismen, von denen die Homologe Rekombination (HR) und die Nicht-homologe Endverknüpfung (*Non-Homologous End Joining* (NHEJ)) zentrale Bedeutung besitzen [54,57,121]. Ein aktuelles Modell besagt, dass die Phosphorylierung von H2AX durch Mitglieder der Phosphoinositol-3-Kinase Familie wie *Ataxia Telangiectasia Mutated* (ATM), *Ataxia Telangiectasia and Rad3 Related* (ATR) und der *DNA-dependent Protein Kinase* (DNA-PK) der Schadensmarkierung dient [19,47]. Zusätzlich werden die freien DNA-Enden durch Bindung der Proteine Ku70 und Ku80 markiert und die DNA-PK rekrutiert. Der entstehende Holoenzymkomplex mit einer aktivierten katalytischen Untereinheit der DNA-PK (DNA-PKcs) vermittelt nachfolgend die Aktivierung weiterer Reparaturproteine wie *Mediator of DNA Damage Checkpoint Protein 1* (MDC1) oder *p53 Binding Protein1* (53BP1) [19,116]. Anschließend binden die Ligase IV und deren Kofaktor XRCC4 (*X-ray repair complementing defective repair in Chinese hamster cells 4*) in Form eines Homotetrameres an den Komplex. Die Autophosphorylierung der DNA-PKcs bewirkt deren Dissoziation,

während die freien DNA-Enden in räumliche Nähe gebracht werden. Schließlich ergänzt die Polymerase µ fehlende DNA-Basen und die Lücken im DNA-Rückgrat werden geschlossen [19,47].

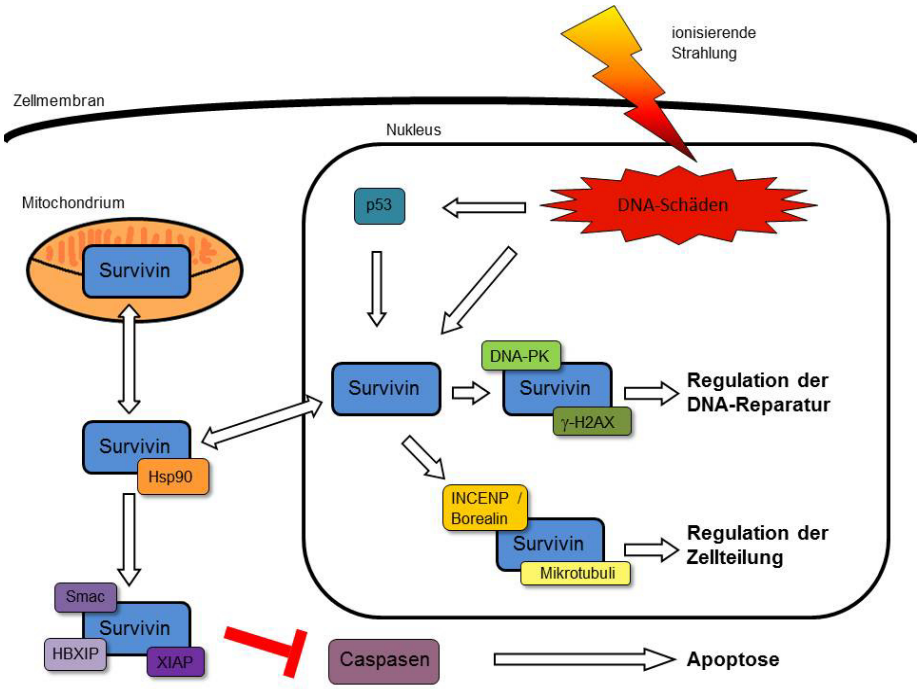

Abbildung 2: *Wesentliche zelluläre Funktionen von Survivin (nach [98]).*
Zytoplasmatisches und aus dem Mitochondrium freigesetztes Survivin hemmt den apoptotischen Zelltod durch Komplexbildung mit dem X-linked Inhibitor of Apoptosis Protein (XIAP) oder Hepatitis B X-interacting Protein (HBXIP) über den Caspase 9 Reaktionsweg, der wiederum negativ durch Second mitochondria-derived activator of caspases (Smac/DIABLO) reguliert wird. Nach DNA-Schädigung durch ionisierende Strahlung transloziert Survivin in den Zellkern und ist an der Regulation der Reparatur von DNA-Doppelstrangbrüchen beteiligt. Nukleäres Survivin ist zudem als essentielle Komponente des Chromosomal Passenger Komplexes (CPC) an der Regulation der Zellteilung und des Zellzykluses beteiligt.

1.3 Klinische Relevanz von Survivin und Bedeutung als molekulares Target

Für eine Vielzahl von Tumorerkrankungen wurde eine Überexpression von Survivin im Tumorgewebe als ein prognostischer Marker für Tumorprogression, aggressivere pathologische Eigenschaften, eine höhere Wahrscheinlichkeit für das Auftreten von Rezidiven bzw. Metastasen beschrieben (Übersicht in [2,8,18,81]).

Neuere Untersuchungen sowohl an prätherapeutischen Biopsien als auch an korrespondierenden Tumor-Operationspräparaten von Patienten mit Rektum- oder Ösophaguskarzinom nach einer präoperativen Radiochemotherapie zeigten zudem eine signifikante

Korrelation zwischen einer erhöhten Survivin-Expression und einem verminderten Gesamtüberleben der Patienten [64,93]. Zudem wurde eine erhöhte Wahrscheinlichkeit zur Metastasenbildung bei fehlender Therapie-induzierter Herunterregulation von Survivin nach Radiochemotherapie beobachtet [12,118]. In diesem Zusammenhang erscheinen auch neuere Untersuchungen von Relevanz, die zeigen konnten, dass ein Survivin-XIAP Komplex an der Aktivierung des Transkriptionsfaktors NF-κB beteiligt ist. Die Aktivierung von NF-κB wiederum führt zur Expression von Fibronektin, welches über einen β1-Integrin, *Focal Adhesion Kinase* (FAK) und Src-Kinase vermittelten Signaltransduktionsweg an Tumorzell-migration, Invasion und Dissemination beteiligt ist [77].

Aufgrund der differentiellen Expression in Tumor- und Normalgewebe und der zentralen Funktion in der Regulation der Apoptose, Aufrechterhaltung der Zellviabilität und Modulation des Therapieansprechens, wurde Survivin schon früh als sinnvolle Zielstruktur einer molekularen Tumortherapie angesehen und entsprechend eine Reihe von Strategien entwickelt, das Protein zu hemmen [81,89]. Diese Strategien umfassen eine molekulare Antagonisierung durch Antisense-Oligonukleotide, Ribozyme und RNA-Interferenz (*small interfering RNA*) sowie die Hemmung von Survivin Funktionen durch „*Small Molecule*“ Inhibitoren, Peptidomimetika, dominant-negative Mutanten oder eine Survivin-basierte Immuntherapie [82,103,119,120].

Die Translation dieser therapeutischen Prinzipien in die klinische Praxis wird derzeit in einer Reihe von Phase I/II Studien evaluiert. Diese klinisch eingesetzten Antagonisten umfassen 2'-O-Methoxy-Methyl modifizierte Antisense-Oligonukleotide (LY2181308: Handelsname Gataparsen, Eli Lilly and Company) und den „*Small Molecule*“ transkriptionellen Inhibitor YM155 (Astellas Pharma). Erste publizierte Ergebnisse zeigen nur eine moderate Wirksamkeit einer Anti-Survivin Monotherapie [44,82,103,119,120]. Es kann jedoch davon ausgegangen werden, dass diese Substanzen in Kombination mit konventionellen Chemotherapeutika und ionisierender Strahlung zu einem verstärkten individuellen Therapieansprechen führen werden. Dennoch ist das Repertoire klinisch einsetzbarer Survivin-Antagonisten noch gering und die Entwicklung neuartiger Hemmstoffe für die klinische Anwendung von hoher Relevanz. Eine Möglichkeit zur Entwicklung innovativer Therapieoptionen könnte in diesem Zusammenhang der Einsatz halbsynthetischer Naturkomponenten darstellen, die eine zunehmende Wertigkeit in der Onkologie erlangen [33,34,131].

1.4 Modulation der Survivin-Expression durch Artesunat

Artemisinin ist ein sekundärer Pflanzenstoff, chemisch ein Sesquiterpen-Lacton, der aus dem Einjährigen Beifuß (*Artemisia annua*) gewonnen wird [61]. Artemisinin wird insbesondere in der traditionellen chinesischen Medizin zur Behandlung von Malaria tropica Infektionen mit multiresistenten Stämmen von *Plasmodium falciparum* eingesetzt [13,61,86]. Strukturell charakteristisch für Artemisinin und seine halbsysnthetischen Derivate wie Artesunat sind ein Trioxanringsystem und eine Peroxidbrücke, die bei hoher Konzentration an Eisenionen instabil wird und in freie Radikale, vor allem Sauerstoffradikale (*Reactive Oxygene Species,* ROS) zerfällt, welche für die therapeutische Aktivität verantwortlich gemacht werden [33].
Interessanterweise zeigte sich jedoch auch, dass die zytotoxische Wirksamkeit von Artemisinin nicht auf den Malariaerreger beschränkt ist, sondern ebenfalls bei unterschiedlichen Tumorzellen beobachtet werden kann. So wurde nach Inkubation mit der Substanz eine Hemmung der Proliferation und Induktion von Apoptose bei einer Vielzahl von Tumorentitäten beobachtet [33,34,79,131]. Des weiteren unterdrückt Artemisinin auch das Wachstum von humanen Tumoren in Xenograftmodellen der Maus und Ratte und führt in klinischen Fallberichten sowie Studien zu einer verbesserten Kontrolle der Tumorprogression und verlängertem Überleben der Patienten [15,22,27,50,68,107,136].
Trotz der zunehmenden klinischen Anwendung sind die molekularen Mechanismen der zytotoxischen Aktivität von Artesunat derzeit erst unvollständig aufgeklärt. Dennoch existieren eine Reihe von Evidenzen, dass die Bildung von ROS und Kohlenstoff-zentrierten Radikalen und nachfolgende Proteinalkylierung bzw. die Induktion von Apoptose und Nekrose wesentlich dazu beitragen [33]. Auf Ebene der Genexpression konnten zudem in genomweiten Expressionsanalysen eine Reihe von Faktoren identifiziert werden, welche die zelluläre Reaktion nach Artesunatbehandlung wesentlich determinieren. Zu diesen Genen zählen Proteine der oxidativen Stressantwort einschließlich von DNA-Reparaturfaktoren, Apoptose und Proliferation regulierende Gene, Onkogene, Tumorsuppressorgene und Angiogenese-assoziierte Faktoren [10,35,76].

1.5 Verbesserte Tumorzellspezifität durch nanopartikuläre Trägersysteme

Ein weiteres aktuelles Konzept zur Individualisierung der Tumortherapie ist die Gentherapie. Dabei werden Nukleinsäuren in den Tumor eingebracht, die selektiv Tumorzell-relevante Faktoren hemmen oder mutierte substituieren um das Tumoransprechen zu verbessern [4,90,101,104].

Eine Option stellt dabei eine DNA (Plasmid)-vermittelte RNA Interferenz (RNAi) in Form einer *short hairpin* RNA (shRNA) oder *micro*RNA (miRNA) dar, die eine spezifische Hemmung („*knock-down*“) ausgewählter Proteine ermöglichen. Eine klinische Anwendung dieser Therapieprinzipien ist derzeit jedoch aufgrund limitierter DNA-Stabilität im Organismus und unzureichender Penetration in die Zielzellen noch eingeschränkt. Als eine Lösungsvariante für diese Probleme wurden deshalb in den vergangenen Jahren unter anderem polymere Nanopartikel aus humanem Serum Albumin (HSA) entwickelt, die eine vielversprechende Klasse von Trägersytemen repräsentieren [65]. HSA als Grundkomponente für die Nanopartikelproduktion besitzt mehrere Vorteile. Zu diesen zählen eine leichte Biodegradation, eine gute Toleranz und geringe Toxizität im humanen Serum, sowie die Möglichkeit vielfältiger Oberflächenmodifizierungen durch das Einfügen funktioneller Gruppen wie Polyethylenglycol (PEG) oder bifunktionellen Maleinimido-Omega-Carboxy Succinimidyl Ester Polyethylenglycol (Mal-PEG-NHS) Crosslinkern [125,132]. Diese Strukturen ermöglichen sukzessive die kovalente Kopplung von Antikörpern mit Spezifität gegen Rezeptoren oder Oberflächenproteinen von Tumorzellen und damit eine optimierte Bindung und erhöhte Penetration der Nanopartikel im Vergleich zu nicht-konjugierten Kontrollen [92,114].

Neben der Kopplung von therapeutischen Antikörpern wie beispielsweise Cetuximab (gerichtet gegen den Epidermalen Wachstumsfaktor Rezeptor EGFR) erscheint auch das *Heat shock protein 70* (Hsp70, aktuelle Nomenklatur HSPA1A) als geeignete Zielstruktur für die Steigerung der Tumorspezifität von Nanopartikeln. Hsp70 vermittelt eine Schutzfunktion in der Zelle gegenüber umweltbedingtem und endogenem Stress und ist exklusiv in einer Vielzahl von humanen Tumoren selektiv in der Plasmamembran nachweisbar [83,105]. Ein Hsp70 positiver Phänotyp ist dabei mit einem aggressiven Tumorwachstum, einem erhöhten Risiko für das Auftreten von Metastasen und einem verminderten Überleben der Patienten assoziiert [83]. Darüber hinaus konnte gezeigt werden, dass die Bestrahlung von malignen

Zellen zu einer vermehrten Translokation von Hsp70 an die Zelloberfläche führt und entsprechend Membran-Hsp70 eine Therapie-induzierbare Zielstruktur darstellt [41]. Aufgrund dieser Charakteristika wurde ein monoklonaler Maus-Antikörper cmHsp70.1 entwickelt, der spezifisch Membran-gebundenes Hsp70 detektiert und bindet [111].

2. Zielsetzung der kumulativen Arbeit

Survivin ist als multifunktionelles Protein an einer Vielzahl von Reaktionskaskaden in der Tumorzelle beteiligt. Darüber hinaus stellt Survivin einen Radioresistenzfaktor dar, dessen Aktivität auf Caspase-abhängigen und -unabhängigen Mechanismen beruht. In diesem Zusammenhang wurde vermutet, dass Survivin auch die Reparatur von strahleninduzierten DNA-Schäden zu stimulieren vermag.

Einen Schwerpunkt der Arbeit bildeten deshalb Untersuchungen zur Klärung der Frage, ob und in welchem Umfang die Strahlenresistenz-vermittelnde Wirkung von Survivin auf der Regulation der DNA-Reparaturkapazität beruht und welche molekularen Mechanismen daran beteiligt sind.

Survivin zielgerichtete Therapiestrategien zur Überwindung eines radioresistenten Phänotyps können zu einer Steigerung der therapeutischen Wirksamkeit einer Strahlentherapie beitragen. Das Repertoire möglicher Survivin-Antagonisten in der klinischen Anwendung ist allerdings noch gering. In weiteren Schwerpunkten der Untersuchungen wurden dementsprechend die Hypothesen geprüft, ob Artesunat, ein halbsynthetisches Derivat der Naturkomponente Artemisinin, einen möglichen Wirkstoff darstellen könnte und in welchem Umfang ein Antikörper gekoppeltes Nanopartikel-Trägersystem zu einer verbesserten Tumorspezifität und Aufnahme von therapeutischen Survivin-miRNA Konstrukten beitragen kann.

3. Zusammenfassung der Ergebnisse

Die Ergebnisse der hier vorgelegten kumulativen Dissertation sind in den nachfolgenden Publikationen dargestellt. Die von mir selbstständig durchgeführten Experimente sowie die Beiträge zu den einzelnen Arbeiten sind im Anhang (Tabelle 1) detailliert aufgeführt.

3.1 Rödel F, **Reichert S**, Sprenger T, Gaipl US, Mirsch J, Liersch T, Fulda S, Rödel C. **The role of survivin for radiation oncology: moving beyond apoptosis inhibition.** Curr Med Chem **2011**;18:191-199.

Dieser Übersichtsartikel gibt den aktuellen Kenntnisstand und Hintergrund zur Rolle von Survivin als Radioresistenzfaktor und sinnvolle Zielstruktur einer therapeutischen Hemmung in Kombination mit Strahlentherapie wieder.

3.2 **Reichert S**, Rödel C, Mirsch J, Harter PN, Tomicic MT, Mittelbronn M, Kaina B, Rödel F. **Survivin inhibition and DNA double-strand break repair: a molecular mechanism to overcome radioresistance in glioblastoma.** Radiother Oncol **2011**;101:51-58.

In dieser Publikation wurde in Glioblastomzelllinien erstmals eine direkte Interaktion von Survivin mit Faktoren der DNA-Doppelstrangbruchreparatur und eine Modulation der DNA-Reparatur als eine weitere Funktion des Proteins gezeigt. Diese Daten liefern eine Bestätigung der Hypothese, dass Survivin eine zusätzliche Komponente in der DNA-Schadensreparatur darstellt.

3.3 **Reichert S**, Reinboldt V, Hehlgans S, Efferth T, Rödel C, Rödel F. **A radiosensitizing effect of artesunate in glioblastoma cells is associated with a diminished expression of the inhibitor of apoptosis protein survivin.** Radiother Oncol **2012**;103:394-401.

Diese Arbeit beschreibt erstmals eine selektive Minderung der Survivin-Expression in Glioblastomzellen durch das halbsynthetische Naturprodukt Artesunat, die wesentlich zu einer strahlensensibilisierenden Wirksamkeit der Komponente beiträgt. Dieses

Ergebnis kann dazu beitragen, längerfristig die therapeutische Effektivität einer Strahlentherapie bei Patienten mit Glioblastom-Tumoren zu verbessern.

3.4 Gaca S, **Reichert S**, Rödel C, Rödel F, Kreuter J. **Survivin-miRNA-loaded nanoparticles as auxiliary tools for radiation therapy: preparation, characterization, drug release, cytotoxicity and therapeutic effect on colorectal cancer cells.** J Microencapsul **2012**, epub ahead of print.

Gegenstand dieses Manuskriptes ist die Etablierung, physikochemische und funktionelle Charakterisierung von, aus humanem Serum Albumin (HSA) hergestellten, Nanopartikeln, die als vielversprechendes Trägersystem für Survivin-miRNA Expressionsplasmide dienen können.

3.5 Gaca S, **Reichert S**, Multhoff G, Hehlgans S, Botzler C, Rödel C, Kreuter J, Rödel F. **Targeting and radiosensitization of glioblastoma cells by cmHsp70.1-antibody coated and survivin miRNA plasmid loaded nanoparticles**; zur Veröffentlichung eingereicht.

Zur Steigerung der Spezifität und zellulären Aufnahme der zuvor beschriebenen Nanopartikel wurde ein Antikörper, der spezifisch membranständiges *Heat shock protein* 70 (Hsp70) auf Tumorzellen detektiert, kovalent an die Partikel gekoppelt. Diese Kopplung führt zu einer Steigerung der zellulären Aufnahme und therapeutischen Wirksamkeit der Nanopartikel und kann entsprechend als Basis für die Entwicklung weiterer alternativer Trägersysteme von Survivin-Antagonisten angesehen werden.

4. Diskussion

Das *Glioblastoma multiforme* ist mit einer Inzidenz von zwei bis drei Neuerkrankungen je 100.000 Einwohner und Jahr der häufigste und aggressivste der astrozytären Hirntumore des Erwachsenen. Trotz intensiver Forschung, verbesserter chirurgischer Techniken (Resektion) und Anwendung einer kombinierten Strahlen- und Chemotherapie ist die Erkrankung mit einer schlechten Prognose assoziiert. So liegt die mediane Überlebenszeit zwischen 11 und 14 Monaten, nur 9% der erkrankten Menschen überleben länger als zwei Jahre [1,42,80]. Das Glioblastom kann somit als ein Paradebeispiel für einen therapieresistenten Tumor angesehen werden, der neuartige Behandlungskonzepte erfordert. Fortschritte werden deshalb insbesondere durch den Einsatz von zielgerichteten molekularen Therapien erwartet, die jedoch ein grundlegendes Verständnis der molekularen und genetischen Grundlagen der Therapieresistenz erfordern.

Für eine Vielzahl von Tumorerkrankungen einschließlich des Glioblastoms wurde eine Überexpression von Survivin als ein prognostischer Marker für ein aggressives Tumorverhalten, Tumorprogression und verkürzte Überlebenszeiten beschrieben [3,96,102,106, 126,130,135]. *In vitro* und *in vivo* Untersuchungen an unterschiedlichen Tumor-Zelllinien und in Tiermodellen zeigten zudem eine enge Korrelation zwischen dem Ausmaß der Survivin-Expression und der zellulären Reaktion auf ionisierende Strahlung. Darüber hinaus gelang durch eine funktionelle, siRNA-, Antisense-Oligonukleotid- oder transkriptionelle Inhibition vermittelte Hemmung von Survivin eine Steigerung der Strahlensensibilität [8,18,20,81]. Die der Vermittlung einer Strahlenresistenz zu Grunde liegende Funktionalität von Survivin ist jedoch vielfältig und beinhaltet Caspase-(Apoptose) abhängige sowie Caspase-unabhängige Mechanismen [11,20,78].

Funktionell wurde Survivin primär als bifunktionelles Protein mit wesentlicher Funktion in der Regulation der Zellproliferation und Apoptose beschrieben [7]. Aktuelle Kenntnisse belegen jedoch, dass das Protein darüber hinaus an vielfältigen Signaltransduktionswegen und molekularen Netzwerken in der Tumorzelle beteiligt ist. Diese reichen von der zellulären Stressantwort, über die Regulation der Angiogenese bis zur Reaktion auf ungünstige Umweltbedingungen und Hypoxie [5,6]. In diesem Zusammenhang spielt die Fähigkeit von Survivin zur Interaktion mit einer Vielfalt unterschiedlicher Bindungspartner und dessen

Lokalisation in unterschiedlichen zellularen Kompartimenten eine entscheidende Rolle für die Funktionalität.

In **Arbeit 3.2** gelang es, nach Bestrahlung von Glioblastomzellen, mittels subzellulärer Fraktionierung, Ko-Immunpräzipitation und Immunfluoreszenzfärbung eine rasche Translokation von Survivin in den Zellkern sowie eine direkte Interaktion des Proteins mit den DNA-Reparaturkomponenten MDC1, γ-H2AX, 53BP1, DNA-PKcs und damit eine neue Funktion von Survivin zu belegen. Während gesicherte Kenntnisse über einen Crm1-vermittelten, aktiven nuklären Export von Survivin existieren, kann aufgrund eines fehlenden nukleären Lokalisationssignals (*Nuclear Localization Sequence*, NLS) über den Mechanismus der raschen nukleären Akkumulation des Proteins nach Bestrahlung bisher nur spekuliert werden [63,115]. So ist zwar aufgrund des geringen Molekulargewichtes von 16,5 kDa eine passive Diffusion denkbar, jedoch aufgrund der schnellen Kinetik der Akkumulation im Zellkern nach Bestrahlung wenig nachvollziehbar. Wahrscheinlicher erscheint deshalb ein aktiver Kotransport von Survivin mit einem oder mehreren Proteinpartnern. Als ein Kandidat wurde in diesem Zusammenhang die *Glykogen Synthase Kinase* 3 β (GSK-3β) identifiziert, die einen stressinduzierten Kotransport ermöglichen könnte [73].

Schon früh wurde die Möglichkeit diskutiert, dass die strahlensensibilisierende Wirkung einer therapeutischen Survivin-Hemmung neben der Steigerung der Apoptosefähigkeit der Tumorzellen auch auf Caspase-unabhängigen Mechanismen beruhen könnte. In diesem Zusammenhang gelang Chakravarti und Mitarbeitern bereits im Jahre 2004 der Nachweis eines möglichen Einflusses von Survivin auf die Reparatur strahleninduzierter DNA-Schäden [20]. Dieser Befund konnte nachfolgend von anderen Gruppen bestätigt werden [18,53,97]. Bisher war jedoch unklar, ob Survivin direkt oder indirekt an den komplexen Mechanismen der DNA-Reparatur beteiligt ist. In der vorliegenden Arbeit wurde erstmals eine direkte Interaktion und Komplexbildung von Survivin mit Komponenten der DNA-Doppelstrangbruch-Reparatur, unter anderem mit Schlüsselfaktoren des NHEJ wie MDC1, γ-H2AX, Ku70 und der DNA-PKcs nachgewiesen. Mechanistisch und funktionell wurden in diesem Zusammenhang nach siRNA-vermittelter Survivin-Hemmung eine verminderte Kinaseaktivität der katalytischen Untereinheit der DNA-PKcs und eine gehemmt Autophosphorylierung des Proteins an Serin 2056 beobachtet. Es kann deshalb angenommen werden, dass Survivin auf einem bisher noch nicht bekannten Mechanismus DNA-PKcs-abhängige Phosphorylierungsvorgänge und nachfolgend die Reparatur von DNA-Schäden zu

steigern vermag. Interessanterweise existieren Parallelen zu der Rolle von Survivin im CPC. In diesem Komplex lokalisiert Survivin mit der katalytischen Domäne der Aurora Kinase B und steigert deren Kinaseaktivität und Affinität zum Substrat Histon H3, während eine Survivin-Hemmung die Kinaseaktivität mindert [100,108]. Entsprechend könnte Survivin eine vergleichbare Funktion in der DNA-Reparatur durch Interaktion mit der DNA-PKcs und γ-H2AX spielen.

Zusammengefasst bestätigen diese Daten die Vorstellung, dass Survivin als Radioresistenzfaktor in Glioblastomzellen die Reparatur von strahleninduzierten DNA-Schäden zu modulieren vermag und eine therapeutische Hemmung zu einer Strahlensensibilisierung führt. Diese Ergebnisse weisen zudem eine zielgerichtete Survivin-Hemmung als vielversprechende Strategie aus, die therapeutische Wirksamkeit einer Strahlentherapie bei Patienten mit Glioblastom zu steigern.

Die zuvor dargestellten Ergebnisse stellen eine weitere Rationale für den klinischen Einsatz von Survivin-Antagonisten zur Strahlensensibilisierung dar und erweitern das Spektrum bekannter Survivin Funktionen. Obwohl derzeit eine Reihe von Strategien wie der Einsatz von Antisense-Oligonukleotiden und „*Small Molecule*“ Inhibitoren als Monotherapie oder in Kombination mit Chemotherapeutika in Phase I/II Studien geprüft werden, besteht weiterhin ein wesentlicher Bedarf an alternativen Wirkstoffen für eine Survivin-vermittelte Tumortherapie [119,120]. Es wurde deshalb in Rahmen der **Arbeit 3.3** die Eignung von Artesunat, einem halbsynthetischen Derivat der Naturkomponente Artemisinin mit bekannter tumorzytotoxischer Wirkung auf die Strahlensensibilität und Survivin-Expression untersucht [33,34]. Dabei gelang es durch Inkubation mit Artesunat in Kombination mit ionisierender Strahlung das klonogene Überleben von Glioblastomzellen zeit- und dosis-abhängig zu supprimieren. Dies korrelierte mit einem Anstieg der Caspase-abhängigen Apoptose, der Induktion eines G2/M Zellzyklusarrestes, einer Hemmung der DNA-Schadensantwort und einer Herunterregulation der Survivin-Expression. Im Gegensatz dazu konnte keine Minderung der Expression von Proteinen wie XIAP, *cellular IAP1* (cIAP1) und cIAP2 beobachtet werden, so dass von einer selektiven Attenuation von Survivin innerhalb der Apoptoseinhibitorfamilie durch Artesunat ausgegangen werden kann.

Ein wesentlicher Mechanismus in der Vermittlung der zellulären Strahlenreaktion ist die Entstehung von Radikalen und reaktiven Sauerstoffmetaboliten (ROS), die Makromoleküle

einschließlich der DNA und Proteine schädigen können [33]. Auch die Wirkung von Artemisinin beruht primär auf einer Eisenionen-abhängigen Spaltung der Peroxidbrücke und anschließender Freisetzung von ROS und Kohlenstoff-zentrierten Radikalen [14,35]. Obwohl der Fokus der Arbeit nicht auf der Rolle von Radikalen lag, existieren überzeugende Daten in der Literatur dafür, dass eine ROS Produktion wesentlich zur zytotoxischen Wirkung von Artesunat in Tumorzellen beiträgt [34,35]. In diesem Zusammenhang wesentlich erscheint eine Arbeit von Kim und Mitarbeitern, die eine signifikante Hemmung der strahlensensibilisierenden Wirkung von Dihydroartemisinin in U373 Glioblastomzellen nach Zugabe des Radikalfängers N-Acetyl Cystein (NAC) nachweisen konnten [60]. Dies belegt einen engen Zusammenhang zwischen der Strahlenantwort und der Artemisinin-vermittelten ROS Produktion.

Aktuelle Untersuchungen zeigen, dass Artesunat die Bildung von DNA-Doppelstrangbrüchen induziert und eine DNA-Schadensantwort triggert, was einen weiteren grundlegenden Mechanismus des zytotoxischen Effektes von Artesunat in Kombination mit ionisierender Strahlung darstellt [14]. Unklar ist in diesem Zusammenhang jedoch, ob Artesunat direkt oder indirekt mit der DNA-Reparatur interferiert. Da, wie zuvor gezeigt, Survivin an der DNA-Schadensreparatur durch Interaktion mit Komponenten der NHEJ-Maschinerie beteiligt ist, kann davon ausgegangen werden, dass das Protein auch an der Vermittlung einer Strahlensensibilisierung durch Artesunat in Glioblastomzellen beteiligt ist [91]. Um einen kausalen Zusammenhang zwischen der Survivin-Expression und Artesunat-Sensitivität zu verifizieren, wurde ein Glioblastom-Zellklon mit stabiler Überexpression eines Survivin-*Green Fluorescent Protein* (GFP)-Konstruktes etabliert und mit Artesunat sowie Bestrahlung behandelt. Dabei gelang nach Survivin-Überexpression eine partielle Rekonstitution des Apoptose-resistenten Phänotyps der Glioblastomzellen und die weitgehende Aufhebung der Artesunat-vermittelten Strahlensensibilisierung. Diese Ergebnisse favorisieren somit einen direkten und kausalen Zusammenhang zwischen der Survivin-Expression und der Artesunat-vermittelten Zytotoxizität und weisen die Substanz als mögliche Option zur Steigerung der therapeutischen Effektivität einer Strahlentherapie bei Patienten mit Glioblastom und anderen Tumorentitäten aus.

Die bisher dargestellten Ergebnisse beziehen sich auf molekulare Mechanismen und mögliche neue therapeutische Strategien einer Strahlensensibilisierung durch Hemmung von

Survivin. Zu einer weiteren aktuellen Klasse von innovativen Behandlungsstrategien zählen auch Nukleinsäure-basierte Therapeutika, die in der Vergangenheit vielversprechende Ergebnisse in präklinischen Untersuchungen sowie im Tiermodell gezeigt haben [66, 124,129]**.** Dennoch stellen eine bisher noch unzureichende DNA-Stabilität, eine unbefriedigende Tumorzellspezifität bzw. Penetration und im Falle von Hirntumoren die Überwindung der Blut-Hirn-Schranke noch unvollständig gelöste Probleme in der klinischen Anwendung dieser Wirkstoffe dar [85,92,114]. Einen möglichen Lösungsansatz bietet die „Verpackung" der Nukleinsäuren in Nanopartikel (NP). Für eine weitere Optimierung der klinischen Verfügbarkeit von Survivin-Antagonisten wurde deshalb ein HSA-basiertes Trägersystem für eine DNA(Plasmid)-abhängige RNA Interferenz (miRNA) mit Spezifität für Survivin etabliert. Diese Partikel wurden anschließend als eine Möglichkeit zur Steigerung der Effizienz einer kombinierten, molekular zielgerichteten Therapie und Strahlentherapie untersucht.

In den "*Proof of Principal*" Untersuchung (**Arbeiten 3.4 und 3.5**) wurde die Eignung der Nanopartikel für eine Anti-Survivin-Therapie und eine Steigerung der Effizienz durch Kopplung eines Antikörpers (cmHsp70.1) gegen die membranständige Form des Hsp70 auf Tumorzellen geprüft. Die Wahl des cmHsp70.1 Antikörpers für die Kopplung an Nanopartikel beruht auf mehreren Evidenzen. Zum einen zeigten umfangreiche Untersuchungen, dass Membran-Hsp70 in Tumorgewebe jedoch nicht im umgebenden Normalgewebe nachweisbar ist [105]. Ein Membran-Hsp70 positiver Phänotyp ist zudem mit hoch aggressiven und metastasierenden Tumoren und entsprechend mit einer schlechten Prognose und verkürztem Überleben der Patienten assoziiert [83]. Zum anderen steigt die Dichte von Membran-Hsp70 nach Bestrahlung in Tumorzellen, jedoch nicht in normalen Zellen an [41]. Die Untersuchungen zeigten dabei eine gesteigerte zelluläre Aufnahme der cmHsp70.1-konjugierten-Nanopartikel, eine verminderte Survivin Proteinexpression, eine Steigerung der Apoptoserate und ein signifikant vermindertes klonogenes Überleben im Sinne einer Strahlensensibilisierung. Diese Ergebnisse stehen in Übereinstimmung mit vergleichbaren Analysen zur Hemmung der Zellzyklus-Kinase *Polo-like kinase 1* nach Inkubation mit Trastuzumab oder Cetuximab-gekoppelten Nanopartikel in *human epidermal growth factor receptor 2* (HER2) bzw. den epidermalen Wachstumsfaktor-Rezeptors (EGFR) überexprimierenden Tumorzellen [74,110].

Schließlich konnte gezeigt werden, dass aufgrund der hohen Austauschrate ein Fluoreszenzfarbstoff markierter cmHsp70.1 Antikörper im Tumorgewebe der Maus aufgenommen und dort bis mindestens 96 Stunden nach einmaliger intravenöser Injektion nachweisbar ist [111]. Die Kopplung des Antikörpers konnte somit auch zu einer gesteigerten Aufnahme der Nanopartikel in Membran-Hsp70-positiven Tumoren beitragen.

Zusammenfassend konnte in diesen Arbeiten gezeigt werden, dass der Apoptoseinhibitor Survivin aufgrund seiner vielfältigen Funktionalität, insbesondere in der Regulation der zellulären Strahlenreaktion und DNA-Reparatur eine ideale Zielstruktur für eine zielgerichtete Tumortherapie in Kombination mit Bestrahlung darstellt. Aktuell existieren jedoch nur wenige Survivin-Antagonisten für den klinischen Einsatz und ein wesentlicher Bedarf an innovativen Optionen zur Steigerung der Tumorzellspezifität und Effizienz der Therapie. Als mögliche Lösungsansätze wurden in der Arbeit eine strahlensensibilisierende Wirkung der halbsynthetischen Naturkomponente Artesunat mit selektiver Hemmung der Survivin-Expression und eine erhöhte zelluläre Aufnahme von Nanopartikel-Trägersystemen nach Kopplung eines tumorspezifischen Hsp70-Antikörpers nachgewiesen. Diese Optionen stellen dabei eine Basis für die Entwicklung alternativer Therapieverfahren und innovativer Trägersysteme für Survivin-Antagonisten im klinischen Einsatz dar.

5. Zusammenfassung

Das *Inhibitor of Apoptosis Protein* Survivin spielt eine prominente Rolle in der Tumorbiologie. Als Paradebeispiel für ein multifunktionelles Protein ist es an der Regulation einer Vielzahl von zellulären Netzwerken, einschließlich der Tumorzellproliferation, der Apoptose und der Antwort auf ungünstige Umweltbedingungen beteiligt. Während es in Normalgewebe nur in wenigen Zelltypen exprimiert ist, findet man in allen bisher untersuchten humanen Tumorentitäten eine Re-Expression des Faktors. Dabei wird dessen Überexpression als prognostischer Marker für ein aggressives Tumorverhalten, eine erhöhte Wahrscheinlichkeit für das Auftreten von Rezidiven und Fernmetastasen sowie ein vermindertes Überleben angesehen. Zudem spielt Survivin eine kritische Rolle in der Vermittlung einer Therapieresistenz von Tumorzellen, die auf der Hemmung von Apoptose und auf Caspase-unabhängigen Mechanismen wie einer Modulation der Zellteilung beruhen.

Ein Schwerpunkt der vorliegenden Dissertation war die Klärung der Frage, ob und in welchem Umfang die Bestrahlungsantwort modulierende Wirkung von Survivin auf der Regulation der Reparatur von DNA-Doppelstrangbrüchen beruht und welche molekulare Mechanismen daran beteiligt sind. Dabei gelang es mittels subzellulärer Fraktionierung, Ko-Immunpräzipitation und Immunfluoreszenzfärbung nach Bestrahlung eine rasche Translokation von Survivin in den Zellkern und eine direkte Interaktion des Proteins mit den DNA-Reparatur- komponenten MDC1, γ-H2AX, 53BP1 und DNA-PKcs zu belegen. Eine siRNA-vermittelte Suppression von Survivin führte zu einer erhöhten Anzahl von Phosphohiston γ-H2AX und 53BP1 Foci als Ausdruck einer gehemmten DNA-Reparatur, während die Überexpression eines Survivin-GFP-Konstruktes eine verbesserte Reparaturkapazität zur Folge hatte. Funktionell korreliert dieses Verhalten mit einer verminderten Autophosphorylierung der DNA-PKcs an Serin 2056 und einer signifikant verminderten Kinaseaktivität. Diese Ergebnisse zeigen, dass Survivin an der Regulation der Reparatur von DNA-Doppelstrangbrüchen beteiligt ist und erweitern das Spektrum der vielfältigen Funktionen des Proteins.

Neuartige, Survivin-assoziierte Therapiestrategien zur Überwindung eines strahlenresistenten Phänotyps könnten zu einer Steigerung der therapeutischen Wirksamkeit einer Strahlentherapie bei Glioblastom-Tumoren beitragen. Es wurden deshalb in einem zweiten

Schwerpunkt ein radiosensibilisierender Effekt von Artesunat, einem halbsynthetischen Derivat der Naturkomponente Artemisinin, in Glioblastomzellen und mögliche zugrunde liegende Mechanismen untersucht. In Kombination von Artesunat mit ionisierender Bestrahlung konnte eine zeit- und dosisabhängige Hemmung der Survivin-Expression beobachtet werden, während die Expression von anderen Mitgliedern der IAP Protein Familie (XIAP, cIAP1, cIAP2) nicht beeinflusst war. Entsprechend der zuvor beschriebenen Funktionalität von Survivin führte dies zu einer gesteigerten Apoptoserate, Induktion eines Zellzyklusarrestes, gehemmter DNA-Reparatur und vermindertem klonogenen Zellüberleben. Diese Ergebnisse geben somit klare Hinweise darauf, dass eine Kombinationstherapie von Artesunat mit ionisierender Strahlung zu einem verbesserten Therapieansprechen beim Glioblastom beitragen könnte und erweitert das Repertoire möglicher Survivin-Antagonisten.

Ein weiteres, bisher noch unbefriedigend gelöstes Problem in der Anwendung von Anti-Survivin-Therapieoptionen ist eine zellspezifische und effektive Applikation der Antagonisten in Tumorzellen. Einen möglichen innovativen Lösungsansatz bietet jedoch die Entwicklung von Nanopartikel-Trägersystemen, die mittels kovalenter Kopplung von Antikörpern gegen tumorrelevante Membranstrukturen/Rezeptoren eine erhöhte therapeutische Wirksamkeit ermöglichen könnten. In einen dritten Schwerpunkt der Arbeit wurde deshalb ein monoklonaler Antikörper (cmHsp70.1) gegen membranständiges *Heat shock protein 70* an humane Serum Albumin-Nanopartikel gekoppelt, die mit Expressionplasmiden für Survivin-spezifische miRNA-Konstrukte beladen waren. Im Vergleich zu einer Isotyp-Kontrolle oder zu plasmidfreien Nanopartikeln konnte nach Inkubation mit cmHsp70.1 konjugierten Nanopartikeln eine signifikant gesteigerte zelluläre Aufnahme und Suppression der Survivin Protein-Expression parallel zu einer erhöhten Caspase3/7 Aktivität, verminderter Zellproliferation und geringerem Überleben beobachtet werden. Die cmHsp70.1 Antikörper konjugierten Nanopartikel können somit als eine Basis für die Entwicklung innovativer Trägersysteme für den Tumorzelloptimierten Einsatz molekular-zielgerichteter Survivin-Antogonisten in der klinischen Anwendung angesehen werden.

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7. Anhang

7.1 Tabelle 1: Auflistung der eigenständig durchgeführten Untersuchungen der Publikationen

Publikation 1	Rödel F, **Reichert S**, Sprenger T, Gaipl US, Mirsch J, Liersch T, Fulda S, Rödel C. **The role of survivin for radiation oncology: moving beyond apoptosis inhibition.** Curr Med Chem **2011**;18:191-199.
Abbildung 1	Diese Abbildung wurde von mir und J. Mirsch erstellt.
Abbildung 2	Die Abbildung fertigte ich zusammen mit J. Mirsch an.
Publikation 2	**Reichert S**, Rödel C, Mirsch J, Harter PN, Tomicic MT, Mittelbronn M, Kaina B, Rödel F. **Survivin inhibition and DNA double-strand break repair: a molecular mechanism to overcome radioresistance in glioblastoma.** Radiother Oncol **2011**;101:51-58.
Abbildung 1 A/B	Der Caspase-Assay sowie der Koloniebildungstest wurden von mir selbständig durchgeführt, ausgewertet und die Abbildungen erstellt.
Abbildung 2 A/B	Der Western Blot wurde von mir angefertigt und die untersuchten Zellen per Immunfluoreszenz angefärbt und ausgewertet.
Abbildung 3 A/B	Zur Untersuchung der Interaktionspartner von Survivin habe ich die Ko-Immunpräzipitationen und die Immunfluoreszenzfärbungen durchgeführt.
Abbildung 4 A/B	Die Kinetik der γ-H2AX-Foci-Bildung wurde vom mir aufgenommen und die Diagramme angefertigt.
Abbildung 5 A/B	Zur Untersuchung der Phosphorylierung der DNA-PKcs wurden die Western Blots von mir erstellt ebenso wie das Diagramm zur DNA-PKcs Aktivität.
Publikation 3	**Reichert S**, Reinboldt V, Hehlgans S, Efferth T, Rödel C, Rödel F. **A radiosensitizing effect of artesunate in glioblastoma cells is associated with a diminished expression of the inhibitor of apoptosis protein survivin.** Radiother Oncol **2012**;103:394-401.
Abbildung 1 A/B/C	Die Untersuchungen zur Bestimmung der Viabilität/Caspaseaktivität ebenso wie die durchflusszytometrischen Analysen des Zellzyklus wurden von mir durchgeführt.

Tabelle 1	Die Ergebnisse der von mir durchgeführten durchflusszytometrischen Bestimmung der Zellzyklusphasen sind in Tabelle 1 zusammengefasst.
Abbildung 2 A/B/C	Zum Nachweis der selektiven Survivin-Inhibition habe ich die Western Blots erstellt.
Abbildung 3 A/B	Die Kinetik der γ-H2AX-Foci-Bildung wurde von mir und V. Reinboldt aufgenommen und die entsprechenden Abbildungen erstellt.
Abbildung 4 A/B	Die Experimente zum klonogenen Überleben der Zellen wurden eigenständig von mir durchgeführt und ausgewertet.
Tabelle 2	In der Tabelle habe ich die strahlenbiologischen Kenngrößen der vorherigen Experimente zusammengefasst.
Abbildung 5 A/B/C	Die Western Blot Analysen, Caspase-Assays und Überlebenskurven nach Plasmid-vermittelter Überexpression von Survivin beruhen auf von mir durchgeführten Experimenten.

Publikation 4	Gaca S, **Reichert S**, Rödel C, Rödel F, Kreuter J. **Survivin-miRNA-loaded nanoparticles as auxiliary tools for radiation therapy: preparation, characterization, drug release, cytotoxicity and therapeutic effect on colorectal cancer cells.** J Microencapsul **2012**, epub ahead of print.
Abbildung 2 B	Die Gelelektrophorese zum Nachweis der Plasmidbeladung wurde von mir durchgeführt und ausgewertet.
Abbildung 3	Zur Analyse der Plasmid-Freisetzung aus Nanopartikel während der Lagerung diente eine Agarose-Gelelektrophorese, die von mir durchgeführt wurde.
Abbildung 6	Den Proliferationstest zur Bestimmung der Toxizität habe ich selbständig durchgeführt sowie ausgewertet.
Abbildung 7 A/B	Die Immuno-Blots und deren densitometrische Auswertung habe ich eigenständig durchgeführt.
Abbildung 8 A-F	Die Experimente zur Bestimmung der Proliferationsminderung wurden aufgrund der großen Probenanzahl gemeinsam mit S. Gaca durchgeführt und ausgewertet.

Publikation 5	Gaca S, **Reichert S**, Multhoff G, Hehlgans S, Botzler C, Rödel C, Kreuter J, Rödel F. **Targeting and radiosensitization of glioblastoma cells by cmHsp70.1-antibody coated and survivin miRNA plasmid loaded nanoparticles.** zur Veröffentlichung eingereicht

Abbildung 2 A/B	Die durchflusszytometrischen Messungen und Fluoreszenzaufnahmen, wurden eigenständig von mir durchgeführt und ausgewertet.
Abbildung 3 A/B	Die Immuno-Blots und die zugehörige densitometrische Auswertung habe ich durchgeführt und dargestellt.
Abbildung 4 A/B	Die Ergebnisse des Caspase- Assays sowie des zum klonogenen Überlebens beruhen auf meinen Experimenten.
Tabelle 2	Die Kenngrößen der klonogenen Überlebenstests wurden von mir in der Tabelle 2 zusammengefasst.

7.2. Publikationen

The Role of Survivin for Radiation Oncology: Moving Beyond Apoptosis Inhibition

F. Rödel*[,1], S. Reichert[1], T. Sprenger[2], U.S. Gaipl[3], J. Mirsch[1], T. Liersch[2], S. Fulda[4] and C. Rödel[1]

[1]*Department of Radiotherapy and Oncology, University of Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany*

[2]*Department of General and Visceral Surgery, University of Göttingen, Robert-Koch-Straße 40. 37075 Göttingen, Germany*

[3]*Department of Radiation Oncology, University Hospital Erlangen, Friedrich-Alexander-University of Erlangen-Nuremberg, Universitätsstraße 27, 91054 Erlangen, Germany*

[4]*Institute for Experimental Cancer Research in Pediatrics, University of Frankfurt am Main, Komturstraße 3a, 60528 Frankfurt am Main, Germany*

Abstract: Alterations in the expression of apoptosis-related proteins, like the inhibitor of apoptosis (IAP) protein family, display a pivotal pathway by which cancer cells acquire resistance to therapeutic treatment. Among this family, survivin, the smallest and structural unique member, deserves growing attention due to its universal over-expression in human tumors, and its prominent role in disparate networks of cellular division, intracellular signaling and apoptosis. Several preclinical studies have demonstrated that targeting survivin expression by the use of small interfering RNAs, dominant negative mutants, antisense-oligonucleotides and small molecule repressors sensitized tumor cells towards chemotherapy and irradiation and reduced tumor growth potential. Due to these properties, survivin has been proposed as a molecular target for anticancer therapies. Recent studies further revealed that radio-sensitization achieved by survivin inhibition seems to be multifaceted and involves caspase-dependent and caspase-independent mechanisms. In general, an enhanced rate of apoptosis, and pronounced cell cycle arrest have been observed. More recently, a hampered DNA-damage response has been noted, indicating a distinct role of the protein in radiation-induced double strand break repair. These properties were linked to a nuclear import and physical interrelationship with members of the DNA-DSB repair machinery such as phospho-histone H2AX and DNA dependent Protein Kinase (DNA-PKcs). The applicability of survivin-driven strategies in clinical practice is currently under investigation as the first survivin inhibitors successfully entered phase I/II trials. Although these trials do not include radiation therapy at present, survivin inhibitors may represent a novel type of molecular antagonists to improve the effectiveness of radiation therapy or chemoradio- therapy.

Keywords: Apoptosis, caspases, DNA-repair, radiooncology, radiosensitization, survivin.

1. INTRODUCTION

Evasion from apoptotic cell death is critical for tumor growth and is reported to be a hallmark of cancer cells [1] and a pivotal mechanism in the resistance to anticancer treatment. Thus targeting the apoptotic pathways by interfering with anti-apoptotic factors may display a promising strategy to counteract resistance, and sensitize cancer cells to anticancer modalities including radiation therapy [2]. Among these anti-apoptotic factors, survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, deserves growing attention due to its universal over-expression in human tumours, and its prominent role in the regulation of a variety of cellular networks, including tumor cell proliferation and adaption to an unfavourable environment [3]. In line with this, it became increasingly clear that the role of survivin in cellular response to anticancer treatment far exceeds a simple inhibition of apoptosis. As a variety of excellent reviews on survivin biology are available [3-6], this review focuses on the role of survivin as a molecular marker and therapeutic target for radiooncological strategies and on the role of survivin in radiation response.

*Address correspondence to this author at the Department of Radiation Therapy and Oncology, University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany; Tel: 0049 696301-6637; Fax: 0049 696301-5091; E-mail: franz.roedel@kgu.de

2. SURVIVIN STRUCTURE AND FUNCTION

Since its discovery in the late nineties survivin, the smallest and structurally unique member of the IAP family, has attracted growing interest for both biologists and physicians due to its unique properties and clinical relevance. The human survivin gene (BIRC5) encodes a 16,5 kD protein of 142 amino residues [7] that is organized as a monomer [8, 9] or stable homodimer [10, 11]. On contrary to the other members of the family, survivin only contains a single baculovirus IAP repeat (BIR) domain, a structural characteristic of all IAPs [10], and an extended carboxy-terminal α-helical coiled-coil domain [12]. Moreover, survivin harbours a variety of phoshorylation sites and interacts with a large number of different protein partners, thus facilitating its multiple activities (Fig. **1**).

In non-cancerous cells, expression of survivin is transcriptionally controlled in a sharp cell cycle-dependent manner, with a marked increase in the G_2/M phase and peak expression in mitosis, that involves CDE/CHR (cell-cycle-dependent/cell-cycle gene homology region) elements located in the survivin promoter [13, 14]. In malignant cells, however, there is evidence that beside cell cycle dependent regulation, survivin may be unregulated independently of mitosis [15, 16] by a variety of mechanisms. These include amplification of the survivin locus on chromosome 17q25 [17], demethylation of the survivin promoter and exons [18], and increased promoter activity [13]. The latter is mediated

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by a variety of oncologic factors such as c-H-RAS [19], c-Myc [20], the developmentally regulated canonical wingless-type MMTV integration site family member WNT/β-catenin/transcription factor 4 (TCF4) [21] or Notch [22]. Furthermore, signal transduction and activator of transcription 3 (STAT3), a transcription factor involved in cytokine signaling [23, 24] and a group of E2F transcription factors [25], which function in the G1/S transition of the cell cycle activate survivin transcription. Moreover, survivin displays a downstream target of nuclear factor-kappa B [26] which, in turn, can be activated by growth factors like insulin like growth factor/mammalian target of rapamycin (mTOR) [27] *via* the phosphatidylinositol 3-kinase/Akt pathway.

Besides its transcriptional activation, wild type tumor suppressor gene p53, non mutated adenomatous polyposis coli gene *(*APC) [28], or phosphatase and tensin homolog deleted from chromosome ten (PTEN) [29] have been shown to decrease survivin expression on the level of transcription. The exact mechanisms of this repression is not entirely clear, it may, however, include binding to the promoter region in the case of p53 [30], changes in chromatin structure promoter accessibility [31], and epigenetic modifications involving DNA cytosine methyltransferase 1 [32]. PTEN suppression is reported to be mediated by direct occupancy of the survivin promoter by forkhead box O1 (FOXO1) and forkhead box O3 (FOXO3a) transcription factors [29] and APC *via* the ß-catenin/TCF4 pathway [33]. Moreover, survivin gene expression was recently linked to main tumor suppression networks in breast cancer involving breast cancer 1 (BRCA1) mediated de novo transcription of silent mating type information regulation 2 homolog 1 (SIRT1), a NAD-dependent histone deacetylase. In turn, SIRT1 bound to the survivin promoter shutting off transcription *via* epigenetic chromatin modifications involving histone H3 [34].

The complexity of survivin regulation is further increased by the presence of alternative splicing of survivin pre-mRNA, resulting in at least four survivin variants: survivin-ΔEx3, survivin-2β [35], survivin-2α [36], and survivin-3β [37]. While wild type survivin is often the predominant transcript, these variants have been reported to differ in cellular localization pattern and to display distinct function in apoptosis regulation [38, 39]. Where as survivin-ΔEx3 and survivin-3β are cytoprotective [40, 41] or modulate the balance between proliferation and cell death by heterodimerization with wt-survivin [42], survivin-2α and survivin-2β display pro-apoptotic properties [35].

Beside its transcriptional regulation, survivin protein is post-translationally regulated by cycles of ubiquitylation [43] and de-ubiquitylation [44], and by multiple phosphorylation events. Known phosphorylation sites comprise Thr34 (cyclin dependent kinase 1), Thr117 (aurora kinase B) and Ser20 (protein kinase A and polo-like kinase 1) [45-48]. These post-translational mechanisms have been largely implicated in survivin protein stability (Thr34, Ser20), and in controlling protein trafficking among various subcellular compartments. Finally, binding of survivin to the chaperon heat shock protein (Hsp) 90 stabilizes the protein and prevents its degradation *via* the ubiquitin-proteasome pathway [43, 49]. On the contrary, inhibition of Hsp90 function or the disruption of the survivin/Hsp90 complex resulted in survivin degradation in acute myeloid leukemia cells [50] where as in other malignant cells (A549, HONE-1 and HT29) Hsp90 inhibition by 17AAG increases the amount of survivin present [51]. Therefore, it seems that inhibition of Hsp90 may not down regulate survivin in certain tumor cells.

Although survivin was primary described to be an bifunctional protein implicated in the regulation of cell proliferation and apoptosis [52], it has now become evident that survivin displays a multifunctional protein that interplays at a crossroad of disparate molecular networks of cellular division, apoptosis, intracellular signaling, and adaption to unfavorable surroundings [3, 9]. In this context, one of the signature features of survivin is its interrelationship with a high number of molecules, including tubulin and various nuclear proteins, Heat shock proteins, a variety of kinases and other members of the IAP family (Fig. **1**). A survivin X-chromosome linked IAP (XIAP) complex for example was recently shown to directly participate in intracellular signaling by activation of the transcription factor NF-κB [64]. NF-κB activation in turn leads to increased fibronectin gene ex-

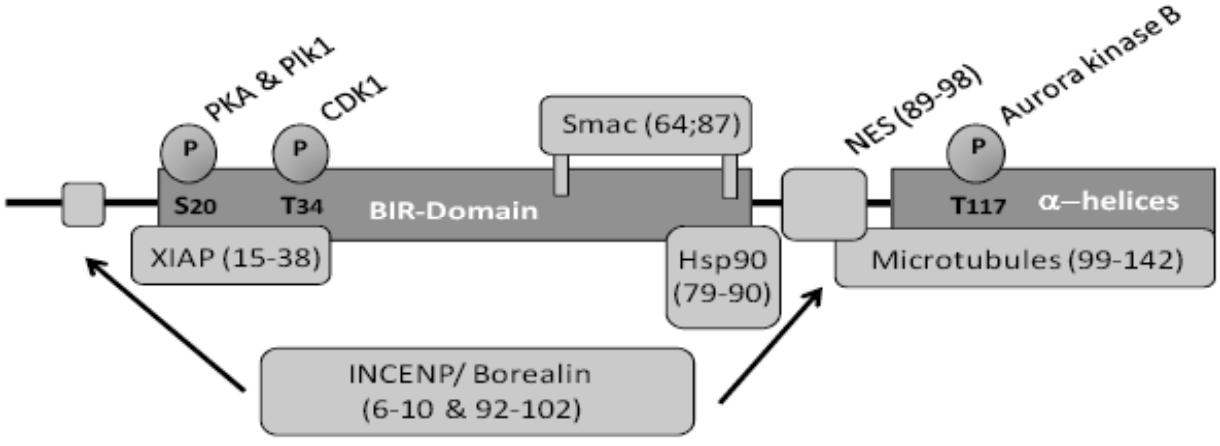

Fig. (1). Schematic representation of survivin protein structure. Functional domains, phosphorylation sites and binding sites for known protein partners are depicted with their correlative residues. BIR: Baculovirus IAP repeat; INCENP: inner centromere protein; NES: nuclear export signal; PKA: protein kinase A; PLK1: Polo-like kinase 1; XIAP: X-linked inhibitor of apoptosis protein; Smac: second mitochondria-derived activator of caspase; Hsp90: 90-kDa heat shock protein.

pression, signaling by beta1 integrins, and activation of cell motility kinases focal adhesion kinase (FAK) and Src to increases tumor cell migration and metastatic dissemination [64].

In the nucleus, survivin acts as an essential member of the chromosomal passenger complex (CPC) composed of the mitotic kinase aurora-B, borealin/dasraB [53] and the inner centromere protein (INCENP) [54]. The CPC plays a key role in coordinating chromosome segregation and cytokinesis [14, 55, 56]. Survivin has been implicated in binding to microtubules of the mitotic spindle, centromeres, kinetochores and intracellular midbodies, enabling coordinated cellular division and is therefore unanimously recognized as an indispensable regulator of cell division [9, 14]. Accordingly, inhibition of survivin expression or disruption of survivin interaction with microtubules induces defective cytokinesis with hyperploidy and multipolar mitotic spindles [57, 58].

Over-expression or targeting of survivin in various cellular systems was clearly associated with inhibition or enhancement of apoptotic cell death [59]. Although convincing evidence exists that survivin inhibits apoptosis, the exact mechanisms are not entirely clear and may result from antagonization upstream of effector caspases. The current thinking is that survivin, in line with most IAPs, except for X-chromosome linked IAP (XIAP), blocks apoptosis by mechanisms other than direct effector caspase inhibition [60] due to the lack of a second BIR domain, which in fact is able to bind caspase 7/9 [10]. Moreover, Survivin associates with the hepatitis B X-interacting protein (HBXIP), and this complex binds pro-caspase 9 and prevents the recruitment of apoptotic protease-activating factor 1 (Apaf-1) to the apoptosome [61]. Additionally, survivin complexes with XIAP *via* its conserved BIR domain and protects it from proteasomal degradation, resulting in a more efficient suppression of caspase-9 activity [62]. Current evidence further suggests that only a pool of survivin compartmentalized in mitochondria and released in the cytosol in response to cell-death stimuli has the ability to associate with XIAP, and this recognition is inhibited by survivin phosphorylation on Ser20 by protein kinase A [9, 46]. Thus survivin-XIAP interaction could be of enormous relevance in the regulation of irradiation induced apoptosis. The survivin-XIAP complex may also reciprocally control survivin stability, as an XIAP-associated molecule, XAF-1, promotes RING-mediated polyubiquitination and proteasomal degradation of survivin [63].

A further mechanism by which mitochondria localized survivin may inhibit apoptosis is by interacting with second mitochondria derived activator of caspase (Smac/Diablo), thus preventing the displacement of bound IAPs from caspases [65]. Interestingly, Smac interacts with both survivin and XIAP, but it only down regulates survivin *via* ubiquitination and proteasomal degradation [66]. The released IAPs may then bind to and inhibit specific caspases [65].

3. SURVIVIN AS DETERMINANT OF RADIATION RESPONSE

Considering a major role of proliferation and apoptosis induction [67] in radiation biology, survivin was early supposed to be involved in radiation response. Indeed, Asanuma *et al.* were the first to report on an inverse relationship between survivin mRNA expression and sensitivity to ionizing radiation. In addition, survivin expression was increased by sublethal doses of irradiation, suggesting a function as an inducible radioresistance factor [68]. A role of survivin as determinant of radiation response was further confirmed in colorectal cancer cell lines of different intrinsic radiosensitivities [69], showing an inverse correlation between survivin expression and apoptotic response to irradiation. Moreover, there is clear evidence that survivin attenuation resulted in a higher amount of cancer cells arrested in G2/M and thus a more radioresponsive phase of the cell cycle [82], indicating that both its function in apoptosis and regulation of cell division may contribute to radiation resistance. Chakravarti *et al.* further demonstrated that survivin expression was much higher in two radioresistant glioblastoma multiforme cell lines (GM20 and GM21) as compared with two radiation sensitive cell lines (GM22 and GM23). Additionally, a pan-cell cycle expression of survivin in the radioresistant cell lines was observed upon radiation exposure [70].

An induction of vascular endothelial apoptosis has been shown to be a major determinant of overall tumor response to radiation therapy [71]. In this context, survivin expression may also contribute to tumor radiation resistance by promoting survival of tumor vascular endothelial cells. Radiation is reported to induce tumor cells secretion of cytokines such as vascular endothelial growth factor (VEGF) [72], which in turn could inhibit vascular endothelial cells apoptosis by up regulating of survivin, as has already been demonstrated for drug-induced apoptosis [73].

4. SURVIVIN AS A NUCLEAR-CYTOPLASMIC-MITOCHONDRIAL SHUTTLE PROTEIN: IMPLICATION FOR RADIATION RESPONSE

A recent advance in the understanding of survivin's function has arisen from the observation that survivin is present at distinct subcellular pools including the nucleus, the cytoplasm, and the mitochondrion [46, 74, 75]. It is widely accepted that the subcellular distribution of survivin plays a distinct role in the ability of the molecule to regulate cell division and survival. While the localization of survivin in the cytoplasm and release from the mitochondrion is considered to be cytoprotective due to its anti-apoptotic activities [5], nuclear localisation of survivin is linked to cell division as a subunit of the CPC [76] (Fig. **2**). In line with this, a leucine-rich nuclear export signal (NES) was described and recent data further indicate that the interaction of the NES with the nuclear export receptor chromosome region maintenance protein 1 homolog (Crm1) is critically involved in survivin intracellular localization and cancer-relevant functions [77-79]. Importantly, subcellular compartmentalization of the protein also plays a role in radiation response, as export-deficient NES-mutants failed to protect tumor cells against radiation-induced apoptosis [80]. Finally, DNA damage by ionizing radiation stimulates a rapid discharge of the mitochondrial pool of survivin in the cytosol that preserves the viability of tumor cells during a protracted G_2 block by antagonizing DNA damage-induced apoptosis [81].

5. NOVEL FUNCTION OF SURVIVIN IN RADIATION RESPONSE

Although there is large evidence that survivin displays a radiation resistance factor in a variety of human malignancies, the underlying molecular mechanisms seem to be multifaceted an involve caspase-dependent and caspase-independent pathways. Chakravarti *et al*. were the first to report on novel, caspase-independent mechanisms by which survivin may enhance tumor cell survival upon radiation exposure [70]. Using an adenoviral vector containing a dominant-negative survivin T-34A mutant, this group report on an impaired DNA repair capacity upon radiation exposure as measured by a comet assay. This was most evident in radiation-resistant primary human glioblastoma cells. More recently, by using phospho-histone γ-H2AX detection as a marker of radiation induced DNA-double strand breaks we and others confirmed a higher incidence of DNA-damage after irradiation in colorectal- and non-small cell lung cancer cell lines after treatment with survivin siRNA or transcriptional repressor YM155, respectively [82, 83].

In mammalian cells, radiation-induced DNA double strand breaks (DSB) are mainly repaired *via* homologous recombination (HR) or nonhomologous end joining (NHEJ) [84]. The latter mechanism directly rejoins the two broken DNA ends using the key components Ku70/Ku80, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), and the ligase IV-XRCC4 complex [85]. Current models implicate that the heterodimeric ku proteins rapidly bind to double stranded DNA ends and recruit DNA protein kinase (PK), generating a DNA-PK holoenzyme complex. An early step in DNA-DSB repair further comprises serine 139 phosphorylation of the histone variant γ–H2AX, by members of the PIKK-family, like ataxia telangiectasia-mutated protein (ATM), ataxia telangiectasia and Rad3 related protein (ATR), and DNA-PKcs [86]. γ-H2AX, visualised as foci at the sites of DNA damage, facilitates recruitment of additional proteins, such as the mediator of DNA damage checkpoint protein 1 (MDC1) [87] or p53 binding protein1 (53BP1) implicated in further signal transduction and regulation of DNA-damage checkpoints [86].

Recently, we could show a nuclear accumulation of survivin following irradiation that was mechanistically linked to DNA-DSB repair. Co-immunoprecipitation analyses and immune- fluorescence co-localisation revealed an interaction between survivin, Ku70, γ-H2AX, MDC1 and DNA-PKcs in nuclear foci. Moreover, survivin knock-down by siRNA confirmed an impaired DNA-DSB repair, as demonstrated by an increased detection of γ-H2AX foci/nucleus at 60 min and a higher amount of residual γ-H2AX foci at 24 hrs post irradiation. Although the function of survivin in the repair complex is not explored in details at present, it may, at least in part result from a hampered Ser2056 autophosphorylation of DNA-PKcs and a significantly decreased DNA-PKcs kinase activity [88]. These data were the first to indicate survivin is linked to DNA-DSB-repair by interaction with members of

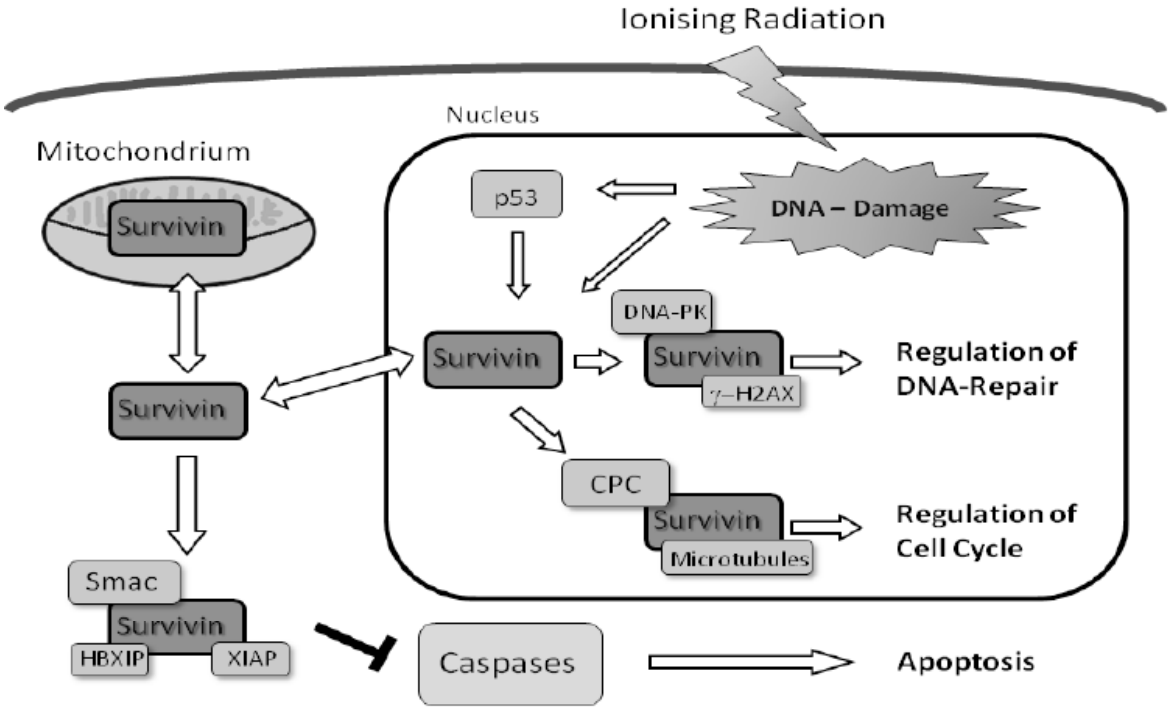

Fig. (2). Schematic presentation of the role of survivin in radiation response. Enhanced expression of survivin mediates radiation resistance of tumor cells through suppression of apoptosis by interfering with caspase activity. The anti-apoptotic activity of survivin is dependent on a CRM1-mediated pathway of nuclear export, as export-deficient survivin mutants failed to protect tumor cells against radiation-induced apoptosis. Besides its role as an inhibitor of apoptosis, survivin also acts as a cell cycle regulatory protein, enabling coordinated cellular division. Accordingly, depletion of survivin alters cell cycle distribution, resulting in a G_2 and mitotic arrest. In addition, survivin appears to be involved in the regulation of DNA-Damage repair by interfering with DNA repair-proteins, thereby enhancing tumor cell survival upon radiation exposure. Please refer to the text for abbreviations.

the DNA-DSB repair machinery, thus regulating DNA-PKcs activity (Fig. **2**).

6. SURVIVIN AS A PREDICTIVE FACTOR FOR TREATMENT RESPONSE TOWARDS RADIOTHERAPY AND RADIOCHEMOTHERAPY

A high expression of survivin in tumor tissues (biopsies or surgical specimens) is commonly associated with an enhanced proliferative index, more aggressive clinicopathologic features and a higher likelihood of tumor recurrence and impaired survival rates in most studies (reviewed in [89-91]).

In patients treated with pre-operative chemoradiation or short-course radiotherapy for rectal adenocarcinoma, survivin expression was inversely correlated with the level of spontaneous apoptosis and was significantly associated with a higher risk of tumor recurrences [92] and inferior survival [93]. Comparable results were obtained from patients with cervical cancers treated with definitive radiotherapy. A high survivin expression was correlated to inferior local control rates and worse overall survival [94, 95]. As tumor-specific expression of survivin is increased by hypoxia [96], the expression of survivin was further investigated in cervical cancer for its relationship to hypoxia parameters. In this context, an inverse correlation with the haemoglobin level and an association with expression of hypoxia-inducible factors 1α (Hif-1α) was observed [94].

A correlation of elevated survivin expression with increased risk of recurrences, lymph node metastases, and shorter survival following radio(chemo)therapy was further confirmed in renal cell cancer [97], non small cell lung carcinoma (NSCLC) [98], T1 bladder carcinoma [99], menigiomas [100], locally advanced prostate cancer [101], and in nasal and paranasal sinus carcinoma [102]. More recent data indicate that intratumoral survivin expression significantly decreased during preoperative chemoradiation in oesophageal and rectal cancer [103]. On the contrary, elevated postoperative survivin levels were highly associated with a higher tumor stage, poor histopathological response, and shortened overall survival.

7. SURVIVIN AS A THERAPEUTIC TARGET FOR RADIATION SENSITIZATION

Due to its differential expression in cancerous and normal tissue and its potential requirement for regulating apoptosis and maintaining cancer-cell viability, survivin was supposed to be a suitable target for molecular tumor therapy [4, 90]. Thus during the last decade, multiple strategies have been employed to target survivin. These strategies comprise molecular antagonisation by the use of antisense oligonucleotides (ASO), ribozymes and small interfering RNAs (siRNAs), suppression of survivin function using small molecule inhibitors and survivin peptidomimetics, interfering with survivin function by the use of survivin dominant-negative mutants and survivin-based immunotherapy.

A radiosensitization by ASO directed against survivin was reported in the H460 lung cancer cells *in vitro* and by increased tumor growth delay of H460 xenografts when combined with radiation [104]. Similar results were reported in the pancreatic cancer cell line AsPC-1 showing that survivin attenuation by siRNA diminished the radio-resistance of AsPC-1 cells [105]. Equally, siRNA or ASO mediated attenuation of survivin in colorectal-, hepatocellular-, non small lung cancer- or epidermoid carcinoma cells increased apoptosis and caspase 3/7 activity after irradiation, which resulted in decreased cell viability and clonogenic survival *in vitro* and decreased tumor growth in xenograft transplant models [82, 106-110]. More recently, Khan *et al.* further reported that down regulation of survivin by the chemotherapeutic drug oxaliplatin diminished radioresistance of head and neck squamous carcinoma cells [111].

Finally, the imidazolium-based small-molecule survivin suppressant YM155 selected *via* a high-throughput screening using a survivin promoter luciferase-assay [112], is reported to sensitize non NSCLC cells to radiation both *in vitro* and delayed the growth of NSCLC tumor xenografts in nude mice to a greater extent than did either treatment with YM155 or irradiation alone [83]. In summary, these preclinical data clearly strengthen the hypothesis that survivin is a suitable molecular target for radiosensitization and display a prerequisite for the clinical application of survivin antagonists in the clinical setting.

8. CLINICAL APPLICATION OF SURVIVIN INHIBITORS

The translation of the pre-clinical findings to the clinic is currently performed with a number of phase I/II clinical trials targeting survivin. These include, among others, the use of 2'-O-methoxy-methyl modified ASOs (LY2181308, Eli-Lilly and Company, Indianapolis, USA) and the low molecular weight molecule inhibitor YM155 (Astellas Pharma Inc., Tokyo, Japan).

Preliminary evidence on the clinical activity of LY2181308 ASO derived from a phase I study in which twenty-four patients with advanced tumors, including breast- or colon cancer and melanoma, were treated with three consecutive daily 3-hr intravenous loading doses (750 mg) followed by weekly maintenance doses [113, 114]. In these patients, 10 % stable disease was reported. Ly2181308 ASO preferentially accumulates in tumor tissue as proven by histochemistry and [11C] Ly2181308 positron emission tomography (PET) resulting in a 20-50 % reduction of survivin protein. Supported by a favorable safety profile, a phase II study of LY2181308 in combination with docetaxel in prostate cancer patients is currently under way [115].

Tolcher *et al.* recently published a phase I study on YM155 including 41 patients with different advanced malignancies. In this trial one complete and two partial responses in three patients with non-Hodgkin's lymphoma, a prostate-specific antigen response in two patients with hormone-refractory prostate cancer, and one minor response in a patient with NSCLC were observed [116]. In another phase I study, Satoh *et al.*, reported on 9 stable disease, and 5 minor responses in 33 evaluable patients [117]. The favorable safety profile with an absence of severe toxicities and the compelling antitumor activity prompted further disease-directed studies of this compound. In these phase II clinical

trials modest single agent activity has been observed with two partial responses and 14 stable diseases in 37 patients with previously treated advanced non-small-cell lung cancer, resulting in a disease control rate of 43 % [118]. On the contrary, only one partial response was reported in 34 patients with unresectable stage II or IV melanoma [119] thus failing to meet its pre-specified primary end-point of two responders in 29 evaluable patients.

CONCLUSIONS AND FUTURE DIRECTIONS

Even though clinical trials targeting survivin for the treatment of cancer are still in their early stage, there is clear evidence that survivin inhibition may not only improve the objective response rates but also possibly circumvent individual treatment resistance. Thus, survivin antagonists may represent a novel type of molecular antagonists to be incorporated in oncological practice either as a single agent or, more likely in combination with established modalities like radiation therapy or chemoradiation. Due to yet not resolved difficulties of drug stability, tumor cell targeting and uptake, it is difficult to predict at present, which therapeutic anti-survivin approach (RNA-Interference, ASO or small molecule inhibitors) will be superior in future clinical strategies. Considering this approaches, however, strategically designing clinical trials and selecting patients that may probably most benefit from survivin inhibition will hopefully improve the therapeutical window by improving tumor response while minimizing tissue side effects.

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DNA damage

Survivin inhibition and DNA double-strand break repair: A molecular mechanism to overcome radioresistance in glioblastoma

Sebastian Reichert [a], Claus Rödel [a], Johanna Mirsch [a], Patrick N. Harter [b], Maja T. Tomicic [c], Michel Mittelbronn [b], Bernd Kaina [c], Franz Rödel [a,*]

[a] Department of Radiotherapy and Oncology; and [b] Neurological Institute (Edinger Institute), University of Frankfurt, Germany; [c] Institute of Toxicology, University Medical Center Mainz, Germany

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ABSTRACT

Background and purpose: Gliomas display prime examples of ionizing radiation (IR) resistant tumors. The IAP Survivin is reported to be critically involved in radiation resistance by anti-apoptotic and by caspase-independent mechanisms. The present study aimed to elucidate an interrelationship between Survivin's cellular localization and DNA damage repair in glioma cells.

Material and methods: Cellular distribution and nuclear complex formation were assayed by immunoblotting, immunofluorescence staining and co-immunoprecipitation of Survivin bound proteins in LN229 glioblastoma cells. Apoptosis induction, survival and DNA repair following IR were assayed by means of caspase3/7 activity, clonogenic assay, γ-H2AX/53BP1 foci formation, single cell gel electrophoresis assay, and DNA-PKcs kinase assay in the presence of Survivin siRNA or over expression of Survivin-GFP.

Results: Following irradiation, we observed a nuclear accumulation and a direct interrelationship between Survivin, MDC1, γ-H2AX, 53BP1 and DNA-PKcs, which was confirmed by immunofluorescence co-localization. Survivin downregulation by siRNA resulted in an increased apoptotic fraction, decreased clonogenic survival and increased DNA-damage, as demonstrated by higher amount of DNA breaks and an increased amount of γ-H2AX/53BP1 foci post irradiation. Furthermore, we detected in Survivin-depleted LN229 cells a hampered S2056 (auto)phosphorylation and a significantly decreased DNA-PKcs kinase activity.

Conclusion: Nuclear accumulation of Survivin and interaction with components of the DNA-double-strand break (DSB) repair machinery indicates Survivin to regulate DSB damage repair that leads to a significant improvement of survival of LN229 glioblastoma cells.

Despite aggressive surgical resections followed by modern radiation therapy and chemotherapeutical protocols, the prognosis for high grade glioma patients still remains poor. The most malignant glioma entity, glioblastoma multiforme, exhibits poor median survival rates in the range of 12–14 months after diagnosis [1,13,25]. The molecular mechanisms underlying therapy resistance in glioma cells remain elusive, however, mounting evidence suggests that preferential activation of DNA damage response checkpoints as well as increased DNA double-strand repair capacity may substantially contribute to this phenomenon [5].

Although Survivin was primarily described to be a bifunctional protein implicated in the regulation of mitosis and apoptosis, it has now been elucidated that Survivin is a multifunctional "nodal" protein that intersects fundamental crossroads of cellular homeostasis including viability and stress response to genotoxic agents [2,3]. Moreover, due to its universal over expression in human tumors and its prominent role in the regulation of a multiplicity of cellular networks, Survivin deserves growing attention as a target of molecular tumor therapy [26,28,29]. Survivin knock down renders tumor cells more sensitive to chemotherapeutic and irradiation treatment [10,20,24,30,42]. In line with this, it became increasingly clear that the role of Survivin in response to ionizing radiation far exceeds a simple inhibition of apoptotic pathways, but involves broader cellular adaptation processes within separate subcellular compartments, possibly including also DNA-damage repair [10,16,17,30].

Exposure to ionizing radiation induces the formation of DNA double-strand breaks (DSBs), resulting in the activation of a complex damage recognition, repair and response machinery [14]. In mammalian cells, DSBs are mainly repaired by two mechanisms, homologous recombination (HR) or non homologous end-joining (NHEJ) [15,39]. Current models of the NHEJ mechanism implicate a rapid binding of the heterodimeric Ku proteins (Ku70/Ku86) to double-stranded DNA ends, and the recruitment of DNA dependent protein kinase (DNA-PKcs), generating a DNA-PK holoenzyme

* Corresponding author. Address: Department of Radiation Therapy and Oncology, University of Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany.
E-mail address: franz.roedel@kgu.de (F. Rödel).

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complex [12,14]. The initial step in DSB repair further comprises phosphorylation of the histone variant γ-H2AX at residue serine 139, by members of the phosphoinositol-3-kinase like kinase (PIKK)-family, e.g. ataxia telangiectasia-mutated protein (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-PKcs [6,9]. Phospho-H2AX (γ-H2AX), focally expressed at the sites of DNA damage and repair, facilitates the recruitment of supplemental repair factors, including the mediator of DNA damage checkpoint protein 1 (MDC1) [36], p53 binding protein 1 (53BP1), and breast cancer 1 protein (BRCA1) implicated in further signal transduction, regulation of DNA damage checkpoints and apoptosis [14,35].

In the present study, we aimed to elucidate the interrelationship between radiation exposure, Survivin's subcellular localization, its interaction with components of the DNA repair machinery, and a possible mechanistic role in the process of DNA–DSB repair. We demonstrate that Survivin physically interacts with the NHEJ DNA repair complex, thus modulating the repair of radiation-induced DSBs.

Material and methods

Cell culture

Human glioblastoma cells LN229 were purchased from the American Type Culture Collection (LGC-Promochem, Wiesbaden, Germany) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% bovine serum (FBS Superior, Biochrom, Berlin, Germany) and 2 mM glutamine at 37 °C, 5% CO_2 and 95% humidity.

Transfection with siRNA and plasmids and irradiation procedure

Transfection of cells with 5 nM (final concentration) Survivin specific siRNA (Ambion, Darmstadt, Germany) as described in detail before [30] and with a control non-silencing siRNA (Qiagen, Hilden, Germany) was performed using the Roti-Fect protocol (Carl Roth, Karlsruhe, Germany). Experimental over expression of Survivin in LN229 cells was conducted by transfection with the plasmid pC3-Surv-GFP coding for a Survivin green fluorescence protein (GFP) construct and control pC3-GFP plasmid at a final concentration of 2 μg/ml using the Roti-Fect protocol. Forty-eight hours after transfection, LN229 cells were irradiated at room temperature with single doses of X-rays ranging from 2 to 8 Gy using a linear accelerator (SL 75/5, Elekta, Crawley, UK) with 6 MeV photons/100 cm focus–surface distance with a dose rate of 4.0 Gy/min.

Subcellular fractionation and immunoblotting

For western-blotting, cells were either lysed in radioimmunoprecipitation assay (RIPA) buffer as previously described [30] or cytoplasmic and nuclear extracts were prepared according to the Nuclear Complex Co-IP kit (Active Motif, Rixensart, Belgium). Equal amounts of protein (10–35 μg) as determined using the micro-BCA-protein assay (Pierce, Rockford, USA) were separated on either 12% SDS polyacrylamide gels or 4–15% gradient gels (Biorad, Munich, Germany) and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were incubated with either anti-Survivin (AF886, R&D Systems, Wiesbaden, Germany), anti-MDC1 (Novus Biologicals, Littleton, CO, USA), anti-DNA-PKcs (clone 4F10C5, Becton Dickinson, Heidelberg, Germany), phospho-specific S2056 DNA-PKcs (ab18356, Abcam, Cambridge, UK), anti-Ku70 (clone 15/Ku70, Becton Dickinson), anti-53BP1 (Novus Biologicals) and phospho-specific S139 H2AX (Upstate Biotechnology, Lake Placid, NY, USA) antibodies followed by appropriate HRP-conjugated secondary antibodies (Santa Cruz, Heidelberg, Germany). Next, blots were developed by an enhanced chemo luminescence detection system (Perkin Elmer, Waltham, USA) and autoradiography (Biomax^R Film, Kodak, Rochester, USA). To confirm equal protein loading and subcellular fractionation, membranes were subsequently reprobed with anti-calnexin (cytoplasm) (Santa Cruz Biotechnology, Heidelberg, Germany) or anti-lamin B1 (nucleus) antibodies (Biozol, Eching, Germany).

Cell cycle analysis

Adherent and detached LN229 cells (1×10^6/ml) were collected by trypsinization and washed with PBS and resuspended in a staining solution containing 1 μg/ml propidium iodide, 4 mmol/L sodium citrate, 1 mg/ml RNAseA (Boehringer, Mannheim, Germany) and 0.1% Triton X-100. FACS analysis was performed with a FACScan apparatus (Becton Dickinson, Heidelberg, Germany) and data were analyzed using the ModFit LT 3.2 software (Verity Software House, Topsham, ME).

Quantification of apoptosis and, caspase-3/7 assay

For quantification of apoptotic LN229 cells, FITC-labeled recombinant chicken AnnexinV (Boehringer Mannheim, Germany) was used in combination with Propidium Iodide to discriminate necrotic cells. In brief, 48 h after irradiation 10^5 cells were resuspended in 500 μl Ringer solution, incubated for 30 min at 4 °C in the dark with 1 μg AxV-FITC/1 μg Propidium Iodide (PI), subsequently analyzed by a FACScan apparatus and CELLQuest^{TM} software (Becton Dickinson). Caspase-3/7 activity was analyzed in a 96 well microplate-format using the CASPASE GLO^{TM}-assay (Promega, Mannheim, Germany) according to the manufacturer's recommendations and quantitated in a luminometer (Berthold, Bad Wildbad, Germany).

Clonogenic survival assay

Following transfection with either Survivin siRNA/control siRNA or pc3-Surv-GFP, LN229 cells were plated in complete DMEM-Medium into culture dishes and irradiated as described above. After 10–14 days, colonies were stained with methylene-blue solution for 30 min and counted. Calculation of survival fractions (SF) was performed using the equation SF = colonies counted/cells seeded × (PE/100), taking into consideration the individual plating efficiency (PE).

Immunoprecipitation

Co-immunoprecipitation of nuclear extracts (500 μg diluted to 500 μl IP Incubation Buffer) was performed using the Nuclear Complex Co-IP kit (Active Motif, Rixensart, Belgium) utilizing Protein A or G Sepharose Fast Flow (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) with anti-Survivin (AF886, R&D Systems), anti-MDC1 (AHP 799, AbD Serotec, Düsseldorf, Germany) and phospho-specific H2AX antibodies (Upstate Biotechnology, Lake Placid, USA). Appropriate isotype control antibodies (Southern Biotech, Birmingham, USA) were used as controls.

Immunofluorescence and quantification of phospho-histone H2AX and 53BP1 foci formation

LN229 cells were cultured on 8-well slides (Thermo Fisher Scientific, Schwerte, Germany) and irradiated with a dose of 2 Gy to assure a discrimination of individual nuclear foci in immunofluorescence staining. Next, slides were fixed with either ice cold methanol or with 3% paraformaldehyde (15 min, RT) as described in [7]. Permeabilization was performed by addition of 0.1% Triton in PBS for 15 min, followed by blocking with Image IT (Invitrogen) and incubation with: anti-Survivin (clone IZC4, DAKO, Hamburg,

Germany), phospho-specific T2609 DNA-PKcs (clone 10B1, Abcam, Cambridge, UK), anti-MDC1 (Novus Biologicals, Littleton, CO, USA), anti γ-H2AX (Upstate Biotechnology) and anti 53BP1 (NB100–304, Novus Biologicals). Primary antibodies were visualized by incubation with appropriate Alexa-labeled secondary antibodies (Invitrogen, Darmstadt, Germany), nuclei were counterstained with DAPI solution (Invitrogen) and coverslips were mounted with Vectashield (Vector Laboratories, Peterborough, UK). Images were taken using an AxioImager Z1 microscope and Axiovision 4.6. software (Zeiss, Göttingen, Germany). In order to quantify γ-H2AX or 53BP1 foci formation for each data point 100–200 nuclei were evaluated.

Single cell gel electrophoresis (comet assay)

Comet assay was performed as described previously [38]. Briefly, at indicated time periods after irradiation, LN229 cells were trypsinized and washed with ice-cold PBS. Next, cells (1×10^4/10 μl) were embedded in 120 μl of low-melting point agarose (0.5% in PBS at 37 °C) onto agarose-coated (1.5% in PBS) slides that were submersed for 1 h in precooled lysis buffer [2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, and 1% Na-laurylsarcosine (pH 7.5), 1% Triton X-100 and 10% DMSO]. Slides were denatured for 25 min at 4 °C in precooled electrophoresis buffer [90 mM Tris–HCl, 90 mM boric acid, 2 mM EDTA] and run at 25 V (300 mA) for 15 min at 4 °C. The ethanol-fixed and dried slides were stained with propidium iodide (50 μg/ml) and analyzed using an image analysis system (Kinetic Imaging Ltd.; Komet 4.0.2; Optilas), determining the Olive Tail Moment (OTM), which represents the percentage of DNA in the tail multiplied by the length between the center of the head and tail of 50 cells per sample [27].

DNA-PKcs activity assay

In order to assess kinase activity, DNA-PKcs-dependent phosphorylation of a biotinylated p53-derived peptide was measured in the presence of [^{32}P-γ]-ATP using a Signa TECT DNA-PK assay kit (Promega, Heidelberg, Germany). Briefly, cells were irradiated, cellular extracts were prepared and incubated with a human Tp53 oligopeptide as substrate in the presence or absence of activated calf thymus. Samples were spotted on a SAM2 biotin capture membrane in duplicates and subsequently read on a phospho-imager (FLA3000, Fuji, Düsseldorf, Germany) and analyzed by an Advanced Image Data Analyzer (AIDA^{TM})-software (Raytest, Germany).

Statistical evaluation

Experimental data are presented as mean ± standard deviations from three or more independent experiments. Levels of significance were calculated using the Student́s *t*-test (Excel® program, Microsoft, Unterschleißheim, Germany).

Results

To analyze whether Survivin expression affects radiation-induced survival and apoptosis, siRNA specific for Survivin and green fluorescence protein (GFP)-tagged Survivin was used to transiently knock down and over express the protein in LN229 glioblastoma cells, respectively. Western blot analysis of total cellular extracts 48 h after transfection revealed a markedly reduced Survivin protein expression in LN229 cells compared to mock- or control-siRNA transfected controls. Overexpression of a Survivin-GFP-construct was proven by the detection of a 43 kDa fusion protein (Supplementary Fig. 1). To analyze whether Survivin inhibition affects spontaneous and radiation-induced apoptosis, we analyzed caspase 3/7 activity and the extent of AnnexinV positive cells 48 h after irradiation. As shown in Fig. 1a and Supplementary Fig. 2, Survivin siRNA transfection resulted in a significant increase ($p < 0.05$) of caspase 3/7 activity and AnnexinV positive LN229 cells as compared to mock- or control-siRNA treated cells and, more pronounced, in cells irradiated with a dose of 2 or 8 Gy. On the contrary, Survivin-GFP over expression caused a significant ($p < 0.05$) decrease in caspase-3/7 activity irrespective of the dose of irradiation, indicating that elevated amounts of Survivin are capable to efficiently suppress caspase-3/7 mediated apoptosis.

Cell cycle analyses performed at 48 h after siRNA transfection and Survivin GFP-over expression (i.e. the time of irradiation in the other sets of experiments) and 8 h after irradiation with a dose of 4 Gy revealed an increased percentage of cells in the G2/M-phase in Survivin siRNA treated LN229 cells (Supplementary Fig. 3), indicating a larger amount of cells in a more radiosensitive stage of the cell cycle.

To further establish a correlation of Survivin expression and radiation responsiveness, clonogenic survival assays were performed in the presence of Survivin-siRNA and Survivin-GFP. Attenuation of Survivin by siRNA shifted down the survival curves for

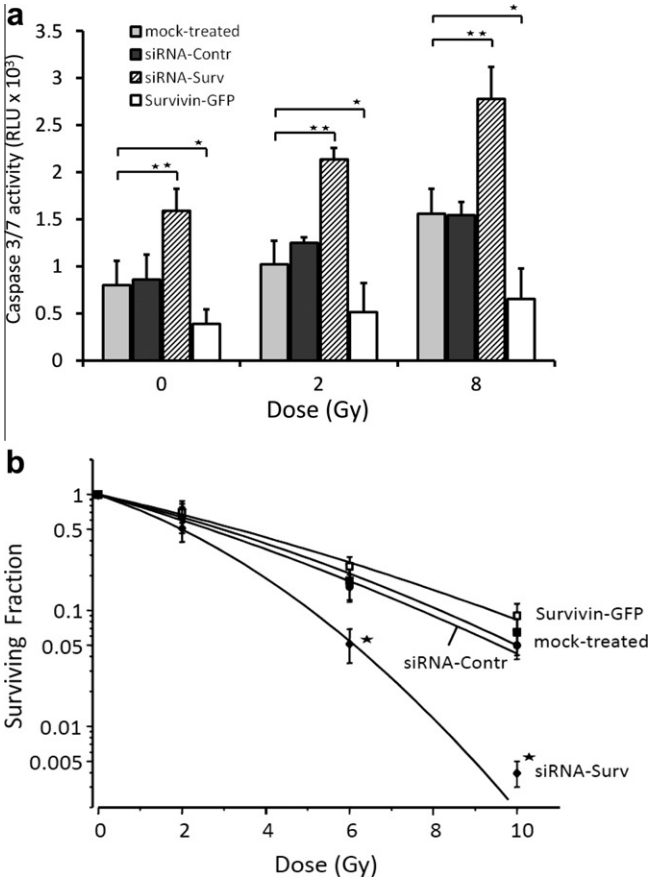

Fig. 1. (a) Analyses of Caspase-3/7 activity following transient transfection with Survivin-specific siRNA oligonucleotides and GFP-tagged Survivin in LN229 glioblastoma cells. At 48 h after transfection cells were irradiated with a dose of 2 or 8 Gy and caspase activity was analyzed 48 h after irradiation. Data are displayed as mean ± SD from three experiments. Asterisks indicate significant differences ($^*p < 0.05$, $^{**}p < 0.01$ as compared to mock-treated cells. (b) Clonogenic survival of LN229 cells transfected with either Survivin-specific siRNA or Survivin-GFP expression plasmid pC3-Surv-GFP. Twenty-four hours later the cells were irradiated with the indicated doses. After 12–14 days, colonies greater than 50 cells were counted and survival curves with survival fractions (SF) normalized to the plating efficiency were fitted according to the linear quadratic equation: $SF = \exp[-\alpha \times D - \beta \times D^2]$ with D = dose. Data are displayed as the mean ± SD from three independent experiments ($^*p < 0.001$ versus mock treated cells).

LN229 significantly, whereas Survivin over expression did not significantly increase survival (Fig. 1b). The 50% and 10% survival rates were significantly reduced ($p < 0.001$) in Survivin siRNA treated LN229 cells, resulting in a calculated radiation-induced cytotoxicity enhancement factor of 1.6 and 1.8, respectively.

A recent advance in the understanding of Survivin's biology has arisen from the observation that Survivin is a nuclear-cytoplasmic/mitochondrial shuttling protein [2,3,34]. In tumor interphase cells, Survivin location is predominantly cytoplasmic, which may, at least in part, depend on the presence of a nuclear export signal (NES), that facilitates an active chromosome region maintenance 1 (CRM1) dependent nuclear export [21,34]. In accordance to this, immunoblottings from subcellular fractionation experiments and immunofluorescence staining in non-irradiated LN229 cells revealed a predominant detection of Survivin in the cytoplasm (Fig. 2). By contrast, 20, 40 and 60 min after irradiation, we observed a nuclear accumulation of Survivin in parallel to a decreased detection in the cytoplasm as demonstrated by immunoblotting (Fig. 2a) and nuclear staining (Fig. 2b).

In order to elucidate and confirm a role of nuclear Survivin in DSB repair, we analyzed whether Survivin may physically interact with members of the DNA damage repair machinery in LN229 cells. Nuclear extracts, obtained at 0, 20, 40 and 60 min after irradiation were co-immunoprecipitated with anti-Survivin antibodies and associated proteins were detected by immunoblotting. As shown in Fig. 3a, DNA-PKcs, MDC1, 53BP1, Ku 70 and γ-H2AX co-immunoprecipitated with Survivin. Exemplary reverse precipitation using either antibodies to MDC1 or γ-H2AX revealed co-immunoprecipitation of Survivin, which further confirmed a complexation. LN229 glioma cells were next subjected to dual immunofluorescence staining, using antibodies to Survivin, DNA-PKcs, MDC1 and γ-H2AX, respectively. Analysis of the merged images by fluorescence microscopy confirmed a co-localization of nuclear Survivin with these proteins in congruent nuclear foci at sites of DNA repair following IR treatment (Fig. 3b).

We next asked whether the shift in cellular compartmentalization of Survivin and complexation with factors of the NHEJ DNA-repair machinery is of mechanistic significance. Therefore, we performed immunofluorescence analysis of γ-H2AX and 53BP1 foci per nucleus at early (0, 20, 40, 60 min) and later time points (2, 6, 12, 24 h) after irradiation in Survivin knockdown LN229 cells. Data are displayed in Fig. 4 and Supplementary Fig. 4, indicating a significant ($p < 0.05$) increase of the amount of γ-H2AX and 53BP1 foci per nucleus in Survivin-depleted cells with maximum values at 60 min after irradiation, and significantly persisting levels at 2–12 h. As depicted in Fig. 4b, compared to mock or siRNA control treated cells, a higher incidence of residual DNA damage was further confirmed by a significant ($p < 0.01$) increase of mean residual γ-H2AX foci per nucleus at 24 h after irradiation. On the other hand, although the data did not reach the level of statistical significance, Survivin-GFP over expression resulted in a lower level of γ-H2AX foci at 2, 8, 12 and 24 h after irradiation. As the γ-H2AX repair foci assay is accepted to prove for DSBs and collapsed replication forks, it may be influenced by chromatin condensation. Therefore, we next performed an independent assessment of DSB induction and repair by the use of the neutral single cell gel electrophoresis (comet) assay. Again, we observed a significantly ($p = 0.02$) higher incidence of non-repaired DSB in cells pre-treated with Survivin siRNA (Fig. 4c).

To understand mechanistically how attenuation of the nuclear Survivin level may impair DNA repair, we next investigated DNA-PKcs S2609 (auto)phosphorylation and DNA-PKcs kinase activity. LN229 cells were exposed to a single dose of 4 Gy in the presence of Survivin siRNA or non-specific control siRNA. Western blot analysis performed without previous irradiation and 20, 40 and 60 min after irradiation with 4 Gy indicated that

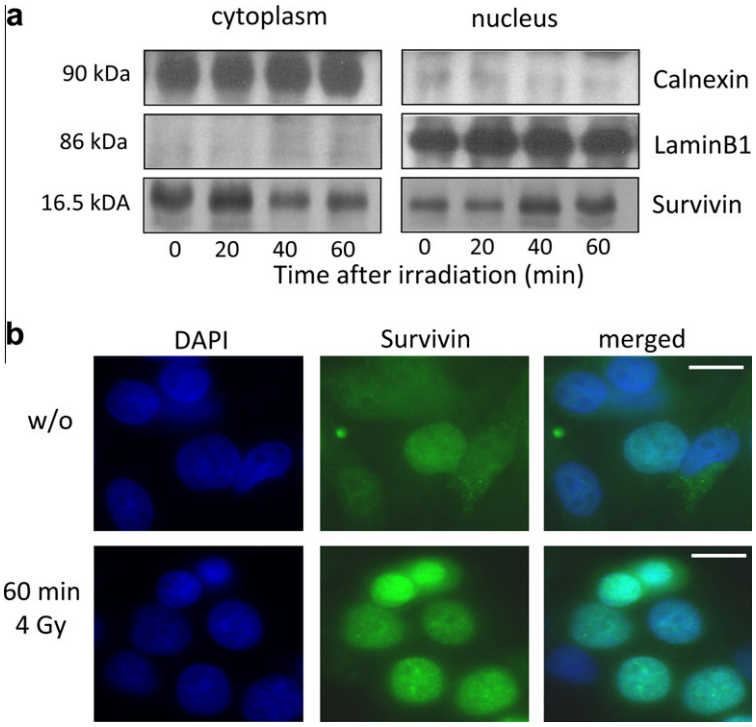

Fig. 2. (a) LN229 cells were irradiated with a dose of 4 Gy. Survivin was detected in cytoplasmic or nuclear extracts by Western blotting (WB) at the indicated time points post irradiation. To confirm equal protein loading and subcellular fractionation, membranes were subsequently reprobed with anti-calnexin or anti-lamin B1 antibodies. b) Fluorescence microscopy analysis of Survivin localization in LN229 cells, mock-irradiated (upper panel) and 60 min after irradiation with 4 Gy (lower panel) using anti-Survivin and Alexa-488 labeled secondary antibodies (green). DNA was counterstained with DAPI (blue). Displayed is one representative out of three repeated experiments (original magnification 630×, bars 10 μm).

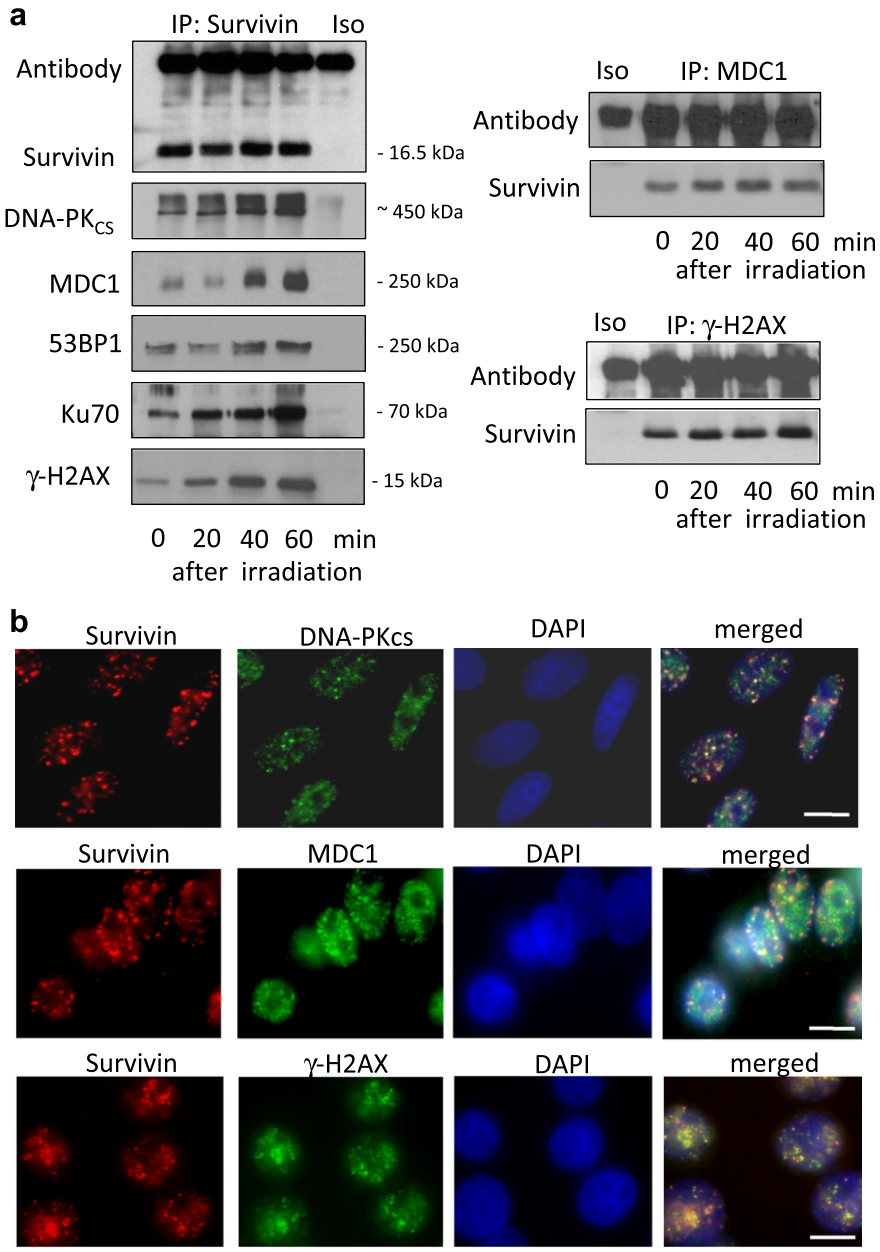

Fig. 3. (a) Nuclear proteins were prepared from LN229 cells at the indicated time points after irradiation with 4 Gy and co-immunoprecipitation (IP) was performed utilizing anti-Survivin antibodies. Associated proteins were next detected by WB. Non specific isotype antibodies (Iso) served as a control. To confirm an association, exemplary anti-MDC1 and anti-phospho histone γ-H2AX antibodies were used for reverse IP and WB for detection of Survivin. (b) Immunofluorescence images of representative cells stained for Survivin and Alexa-594 conjugated secondary antibody (red), DNA-PKcs (upper panel), MDC1 (middle panel) and γ-H2AX (lower panel) and Alexa-488 conjugated secondary antibody (green) and DNA (DAPI staining, blue) 40 min after irradiation of LN229 cells with 4 Gy. (Original magnification 630×, bars 10 μm).

Survivin attenuation by siRNA resulted in a reduced level of S2056 (auto)phosphorylation of DNA-PKcs (Fig. 5a), indicating a distinct role of Survivin in the regulation of DNA-PK enzymatic activity. To further proof this, DNA-PKcs dependent phosphorylation of a biotinylated p53-derived peptide was measured in the presence of [^{32}P-γ]-ATP using a Signa TECT DNA-PK assay. In mock-treated or siRNA-control transfected LN229 cells DNA-PK activity gradually increased with time, reaching the highest value at 40 min after irradiation. By contrast, in Survivin-siRNA treated LN229 cells a significantly ($p < 0.001$) lower DNA-PK activity was observed (Fig. 5b). Notably, Survivin-GFP overexpression did not further increase DNA-PK kinase activity, indicating a putative saturation effect.

Discussion

Survivin was primary described as a bifunctional protein implicated in the regulation of cell proliferation and apoptosis [4]. Now, however, it becomes evident that Survivin possesses multifunctional properties that interplay at a crossroad of various molecular networks of cellular division, apoptosis, and stress adaption to unfavorable exposures [2,3]. One of the most important features of Survivin is its interrelationship with a growing number of molecules, including tubulin and various nuclear proteins. Moreover, a recent advance in the understanding of Survivin's function and biology has arisen from the observation that Survivin is present at distinct pools including the nucleus, the cytoplasm,

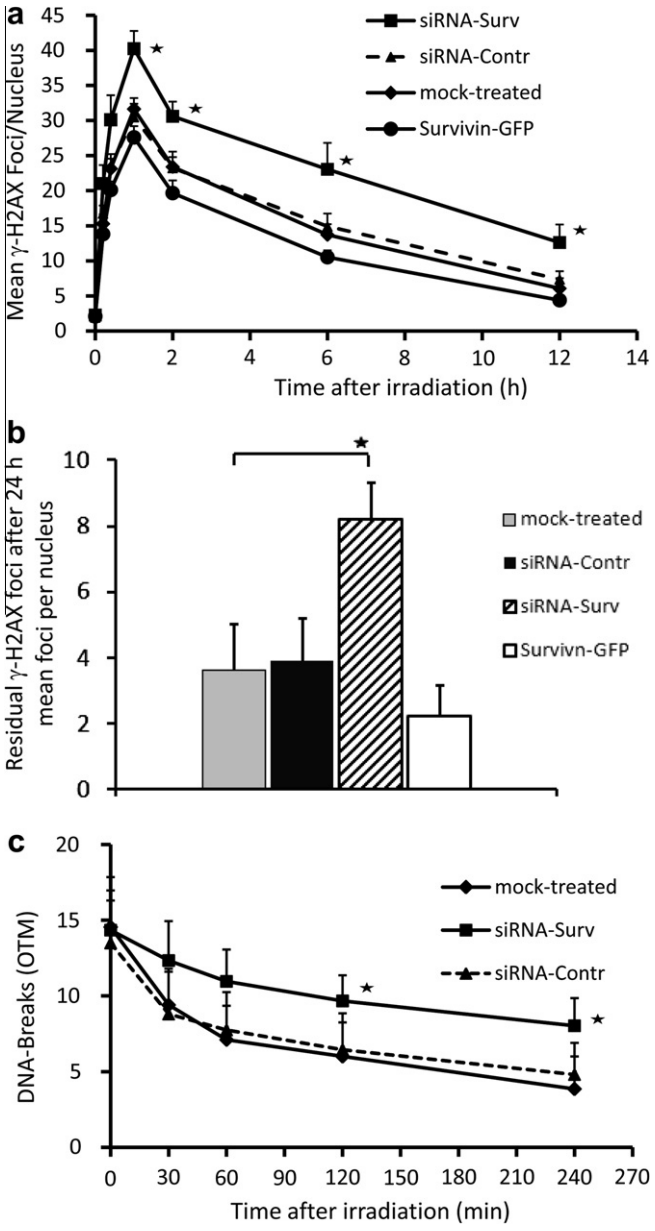

Fig. 4. (a) Detection of serine 139 phosphorylated histone γ-H2AX following treatment with Survivin-specific siRNA and GFP-tagged survivin. Forty-eight hours after transfection, cells were irradiated with a dose of 2 Gy to assure foci discrimination and subsequently stained using γ-H2AX antibodies, Alexa-594 secondary antibody and DAPI for nuclear counterstaining. Data are given as mean ± SD from three repeated experiments ($^*p < 0.05$ versus mock-treated cells). (b) Residual DNA damage at 24 h after irradiation was visualized by using γ-H2AX. Each bar represents the mean ± SD of residual repair foci per cell nucleus. For each data point 50–100 nuclei were evaluated. ($^*p < 0.01$ versus mock treated cells). (c) At the indicated times after irradiation LN229 cells were subjected to a single cell gel electrophoresis (comet) assay. Data are given as the Olive Tail Moment (OTM) ($^*p = 0.02$ versus mock treated cells).

the mitochondrion [2,34] and, more recently, the extracellular space [19]. The localization of Survivin in the cytoplasm is suggested to be cytoprotective because of its anti-apoptotic function, whereas its nuclear localization controls cell division as a subunit of the chromosomal passenger complex (CPC) implicated in chromosome segregation and cytokinesis [3,32].

In the present study, we confirmed nuclear accumulation and complexation of Survivin with the DNA repair proteins MDC1, DNA-PKcs and Ku70 as well as the phosphorylated histone H2AX, suggesting a role or involvement of Survivin in DSB repair following exposure to ionizing radiation [7]. Survivin is reported to structurally contain a nuclear export signal (NES) that facilitates an active CRM1-dependent nuclear export implicated in the predominant cytoplasmic localization of Survivin in tumor cells [21,34]. On the contrary, no nuclear localization signal (NLS) is present in the Survivin sequence to facilitate a karyopherin receptor mediated nuclear import [40]. Moreover, the rapid kinetic of nuclear accumulation is unlikely to arise from a passive diffusion of the 16.5 kDa protein, but may be mediated by a more specific and active shuttle mechanism. Notably, recent studies revealed a complex of Survivin with Glycogen Synthase Kinase 3beta (GSK3ß) that facilitates a nuclear shuttling following stress-induced translocation of GSK3ß to the nucleus [22]. Thus GSK3ß may also display one putative nuclear shuttling partner for Survivin following irradiation.

Although it has been convincingly shown in preceding experiments that Survivin is a radiation resistance factor in a variety of cancer cells, including glioblastoma [10], the underlying molecular mechanisms are complex and far exceed a simple inhibition of irradiation-induced apoptotic cell death [8,31]. In line with this, Chakravarti et al. were the first to report on caspase-independent mechanisms by which Survivin may enhance tumor cell survival upon radiation exposure [10]. Using an adenoviral vector containing a dominant-negative Survivin T-34A mutant, this group reported on an impaired DNA repair capacity upon radiation exposure as analyzed by a neutral comet assay. Using the γ-H2AX foci formation assay, a hampered DNA damage repair was recently confirmed in colorectal cancer [7,30] and in non-small cell lung cancer cell lines in the presence of Survivin specific siRNA or YM155, a small molecule inhibitor of Survivin expression [16]. It remains, however, elusive whether Survivin is directly or indirectly involved in the complex repair processes following radiation therapy.

Here we propose a direct function of nuclear Survivin in DNA damage repair by physical interaction with the NHEJ repair proteins MDC1, DNA-PKcs and Ku70. Interestingly, we also found Survivin interacted with γ-H2AX, a histone modification that supports the recruitment of repair proteins to the site of damage. Exposure to ionizing radiation induces the formation of DSBs, resulting in the activation of a complex damage recognition, repair and cellular response machinery [14]. Upon irradiation-induced DNA damage, MDC1 is rapidly re-located to sites of DSB acting as a scaffold for the recruitment and accumulation of additional repair proteins, thereby mediating amplification of DNA damage signaling. MDC1 is reported to directly bind γ-H2AX and DNA-PKcs and to facilitate DNA-PKcs dependent repair processes by regulating its (auto) phosphorylation [23,37]. We, therefore, speculated that γ-H2AX-MDC1-Survivin-Ku70-DNA-PKcs complexation may facilitate DNA-PKcs enzymatic activity following irradiation. Indeed, we observed a lower level of S2056 (auto)phosphorylation of DNA-PKcs and a significantly decreased DNA-PKcs kinase activity in Survivin knockdown LN229 cells. These data suggest a new function of Survivin, i.e. besides its well-characterized role as caspase inhibitor and indispensable subunit of CPC [32,33] regulating DSB repair by stimulating non-homologous end-joining. The protective role of Survivin in IR-induced cell death, which was confirmed in this work as well, may thus be explained both by caspase inhibition and by stimulation of DNA repair. The exact molecular mechanism(s) of the interaction of Survivin with players of the NHEJ machinery, however, remains to be established. It may originate from a direct Survivin-DNA-PKcs association that has impact on DNA-PKcs kinase activity or indirectly by interfering with early processes involved in DNA damage repair, e.g. the formation of γ-H2AX foci, the assembly of the MRE11-RAD50-NBS1 (MRN) complex or activation of the ATM kinase.

Notably, there are some parallels to the role of Survivin in the CPC [32,41]. In this complex, the protein localizes to the catalytic

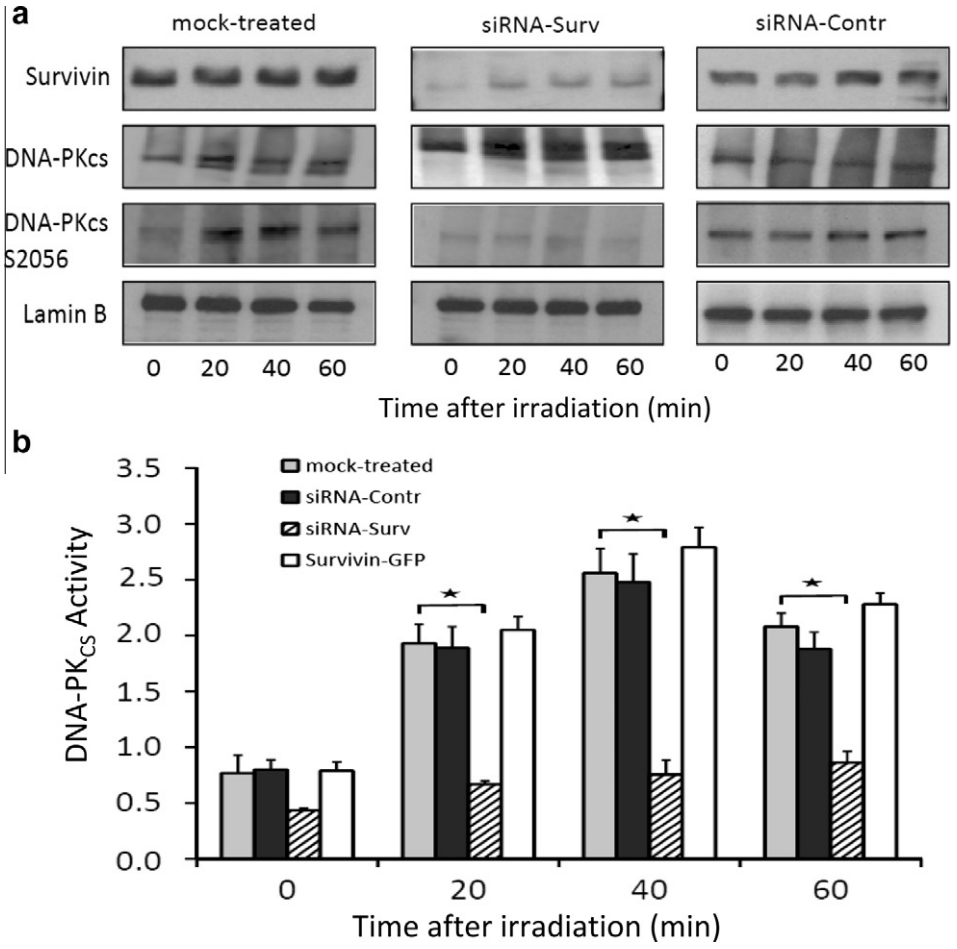

Fig. 5. (a) Nuclear extracts were prepared from LN229 cells at the indicated time points and Survivin, DNA-PK total protein and S2056 phoshorylated DNA-PK were assayed by western blotting after transfection with survivin-specific siRNA and irradiation with 4 Gy. Mock-treated and non specific-siRNA treated cells served as a control, lamin B1 for equal protein loading. (b) To assess kinase activity, DNA-PK-dependent phosphorylation of a biotinylated p53-derived peptide was measured in the presence of [32P-γ]-ATP using a Signa TECT DNA-PK assay. Data are presented as mean ^{32}P-activity ± SD from three repeated experiments. ($^{*}p < 0.001$ versus mock-treated cells).

domain of the mitotic kinase Aurora-B, enhancing its kinase activity both *in vitro* and *in vivo*, and targets Aurora-B to its substrate histone H3 [11]. On the contrary, Survivin knock down cells display lower Aurora-B kinase activity. More recently, an evolutionary conserved binding pocket in the baculovirus inhibitor of apoptosis (BIR) domain of Survivin was reported to recognize phosphorylated histone H3, thus mediating recruitment of the CPC to chromosomes and activating its kinase subunit Aurora B [18]. Thus, Survivin may play a putative analogical axillary function for DSB-repair by interfering with γ-H2AX and kinase DNA-PKcs.

In conclusion, our data support the view that Survivin displays a radiation resistance factor in glioblastoma. This is, at least in part, explained by its role as a factor that stimulates radiation-induced DNA damage repair. This may well contribute to the "nodal" properties of Survivin [3] and may impact on the regulation of disparate networks of cellular adaption to genotoxic stress. Moreover, our data indicate that attenuated DNA repair following down regulation or inhibition of Survivin goes along with a sensitisation in glioblastoma cells that are otherwise resistant to killing by irradiation. This concept further suggests that targeting Survivin in high grade gliomas may be a promising strategy to increase the therapeutic ratio of radiation therapy in future clinical trials.

Conflicts of Interest

There are no actual or potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.06.037.

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Radiotherapy and Oncology 103 (2012) 394–401

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Molecular radiobiology

A radiosensitizing effect of artesunate in glioblastoma cells is associated with a diminished expression of the inhibitor of apoptosis protein survivin

Sebastian Reichert [a,1], Vera Reinboldt [a,1], Stephanie Hehlgans [a], Thomas Efferth [b], Claus Rödel [a], Franz Rödel [a,*]

[a] Department of Radiotherapy and Oncology, University of Frankfurt am Main; and [b] Department of Pharmaceutical Biology, University of Mainz, Germany

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ABSTRACT

Background and purpose: Novel strategies to overcome an irradiation resistant phenotype may help to increase therapeutic efficacy in glioblastoma multiforme. The present study aimed to elucidate radiation sensitizing properties of artesunate, a semi synthetic derivate of artemisinin and to assess factors involved in this effect.

Materials and methods: LN229 and U87MG cells were treated with various concentrations of artesunate and radiation response was determined by a colony forming assay. Cell numbers, apoptosis induction, cell cycle distribution, and DNA repair following combined modality treatment were monitored by MTT-, caspase 3/7 assay, cytofluorometry, and γ-H2AX foci formation. Expression of survivin, survivin–GFP fusion protein, XIAP, cellular (c)IAP1 and cIAP2 was monitored by Western immunoblotting.

Results: Treatment of glioma cells with artesunate and irradiation resulted in an increased apoptotic fraction, pronounced G2/M arrest and increased DNA damage as demonstrated by an elevated amount of γ-H2AX foci/nucleus. Incubation with artesunate lowers survivin expression in a time and dose-dependent manner, whereas expression of XIAP, cIAP1 and cIAP2 was not affected. In clonogenic assays, treatment with artesunate revealed a significantly reduced surviving fraction, whereas stable over expression of a survivin–GFP protein reversed artesunate-mediated radiosensitization.

Conclusion: Artesunate selectively down regulates survivin that contributes to a radio-sensitization of glioma cells by an increased induction of apoptosis, cell cycle arrest, and a hampered DNA damage response.

 Radiotherapy and Oncology 103 (2012) 394–401

Despite aggressive surgical procedures followed by radiation and chemotherapy, the prognosis for glioblastoma multiforme (GM) patients still remains poor with median survival rates in the range of 12–14 months after diagnosis [1,21,35]. From a therapeutical point of view, glioblastoma displays a prime example of a therapy resistant tumor which may be explained by the presence of tumor cells with a radiation- and chemo-resistant phenotype in line with an increased DNA repair capacity [7]. Thus, there is a critical need to develop new anticancer drugs to increase responsiveness of glioma tumors.

Artesunate is a semisynthetic derivate of artemisinin, a sesquiterpene lactone which was isolated from the plant *Artemisia annua* and used in traditional Chinese medicine to treat fever and chills [29]. During the last decades, however, artemisinin and its derivatives have gained considerable interest as a new generation of anti malarial drugs [41] and have been proven to display distinctive cytotoxic activity in a variety of tumor cells [16,17,33,47]. Moreover, artemisinin in vivo suppresses the growth of human tumor cells in xenograft models in rats and mice [13,14,25,30,36] and has been confirmed to be beneficial in controlling disease progression and prolonging survival in clinical case reports and trials [9,42,48].

Despite its growing impact in therapy, molecular mechanisms and sequence of events underlying artesunate's anti cancer efficacy are still not resolved in full detail. However, mounting evidence has been accumulated, that generation of reactive oxygen species (ROS) or carbon-centered radicals and subsequent protein alkylation and downstream mechanisms like induction of apoptotic/necrotic cell death are involved in these processes (reviewed in [16]). Moreover, using microarray expression analysis, a variety of genes have been identified to significantly affect the response of tumor cells to artemisinin [15,20]. These genes comprise factors involved in the oxidative stress response, including DNA damage and repair genes, apoptosis regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes, and angiogenesis-related genes [4,15].

Alterations in the expression of apoptosis-regulating proteins, like the family of inhibitor of apoptosis proteins (IAPs), display a hallmark of cancer cells for acquired resistance to therapeutic treatment [2,22]. Among this group, the smallest member survivin

* Corresponding author. Address: Department of Radiotherapy and Oncology, University of Frankfurt am Main, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany.
E-mail address: franz.roedel@kgu.de (F. Rödel).

[1] Both authors contributed equally to this work.

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deserves growing attention due to its prominent role in disparate networks of cellular division, intracellular signaling and apoptosis [3]. Several preclinical studies have demonstrated that targeting survivin expression by RNA interference, antisense-oligonucleotides (ASO) and small molecule repressors sensitized tumor cells toward irradiation and reduces tumor growth potential [27,40]. Recent data further indicate that a radiosensitizing effect of survivin inhibition seems to be multifaceted and involves increased apoptosis and caspase-independent mechanisms. The latter comprise induction of a G2/M cell cycle arrest and a distinct role of survivin in the regulation of radiation-induced double-strand break repair [11,12,26,37].

In the present study, we aimed to investigate radiation sensitizing properties of artesunate on glioblastoma cells and to assess possible mechanisms and factors involved in this effect. Our results show that artesunate down regulates the IAP survivin and enhances radio-responsiveness of glioma cells by an increased level of apoptosis, a hampered DNA damage repair and as a consequence decreased clonogenic survival.

Materials and methods

Cell culture

Human glioblastoma cell lines LN229 and U87MG were obtained from the American Type Culture Collection (LGC-Promochem, Wiesbaden, Germany), and were either cultured in Dulbecco's Modified Eagle's Medium (DMEM: LN229) or Minimum Essential Medium (MEM: U87MG) (both, Sigma–Aldrich, Munich, Germany) supplemented with 10% or 20% fetal bovine serum (PAA, Coelbe, Germany), 1 mM glutamine and 1% penicillin/streptomycin (Biochrom, Berlin, Germany). The environmental conditions were 37 °C, 5% CO_2 and 95% humidity.

Treatment with artesunate and irradiation procedure

LN229 and U87MG cells were plated in cell culture flasks 24 h before treatment to reach a confluence of 80–90%. Artesunate (Sigma–Aldrich, Munich, Germany) was dissolved in DMSO and a stock solution of 50 mg/ml was prepared. Artesunate was added to the cell culture medium at a final concentration ranging from 1 to 64 μg/ml and incubated continuously for 24 h. Irradiation was performed at room temperature with single doses of X-rays ranging from 2 to 10 Gy using a linear accelerator (SL 75/5, Elekta, Crawley, UK) with 6 MeV photons/100 cm focus–surface distance with a dose rate of 4.0 Gy/min.

Quantification of apoptosis and caspase-3/7 assay

For quantification of apoptotic LN229 and U87MG cells, subG1 content was analyzed following staining of the cells with 1 μg/ml propidium iodide, 4 mmol/l sodium citrate, 1 mg/ml RNaseA (Boehringer, Mannheim, Germany) and 0.1% Triton X-100 by a FACScalibur apparatus and CELLQuest™ software (Becton Dickinson, Heidelberg, Germany). Caspase-3/7 activity was analyzed in a 96 well microplate-format using a CASPASE GLO™-assay (Promega, Mannheim, Germany) according to the manufacturer's recommendations and quantitated using a luminometer (Berthold, Bad Wildbad, Germany).

3-(4,5-Methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Cells were plated at a density of 2–10 $\times$ 10^3 cells/200 μl in a 96-well microplate, grown for 6 h and subsequently exposed to artesunate (24 h) and irradiation. After an additional 48 h of incubation at 37 °C, MTT (Applichem, Darmstadt, Germany) was added (20 μl/well of a 5 mg/ml solution in PBS) for 4 h. Solubilization of the converted purple formazan dye was accomplished by adding 50 μl/well of 0.01 N HCl/20% SDS and incubation overnight at 37 °C. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA reader (VIKTOR™ 1420, Waltham, MA, USA).

Cell cycle analysis

Adherent and detached LN229 and U87MG cells (1×10^6/ml) were collected by trypsinization, washed twice with PBS and resuspended in a staining solution containing 1 μg/ml propidium iodide, 4 mmol/l sodium citrate, 1 mg/ml RNaseA and 0.1% Triton X-100. FACS analysis was performed with a FACScalibur apparatus (Becton Dickinson) and quantification was performed using CELLQuest™ software (BD).

Immunofluorescence and quantification of phospho-histone γ-H2AX foci formation

Glioblastoma cells were cultured on 8-well slides (BD Falcon, Heidelberg, Germany), treated with artesunate and irradiated with a dose of 2 Gy to assure a discrimination of individual nuclear foci in immunofluorescence staining. Slides were next fixed with either ice cold methanol or with 3% paraformaldehyde (15 min, room temperature: RT) as described in [11]. Permeabilization was performed by addition 0.1% of Triton in PBS for 15 min, followed by blocking with 5% BSA, 0.05% Triton X-100, 1 μg/ml mouse/rabbit serum and incubation with anti γ-H2AX (Upstate Biotechnology, Lake Placid, USA) primary antibodies. Next, binding was visualized by incubation with appropriate Alexa-labeled secondary antibodies (Invitrogen, Darmstadt, Germany), nuclei were counterstained with DAPI solution (Invitrogen) and coverslips were mounted with Vectashield (Vector Laboratories, Peterborough, UK). Images were taken using an AxioImager Z1 microscope and Axiovision 4.6. software (Zeiss, Göttingen, Germany). In order to quantify γ-H2AX foci formation for each data point 150–200 nuclei were evaluated from three independent experiments.

Immunoblotting

For Western immunoblotting, cells were washed with PBS and lysed in radioimmuno-precipitation assay (RIPA) buffer supplemented with protease inhibitors as previously described [11]. Equal amounts of protein (10–35 μg) as determined by a micro BCA-protein assay (Pierce, Rockford, USA) were separated on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were next incubated with either anti-Survivin (AF886, R&D Systems, Wiesbaden, Germany), anti-XIAP, anti-GFP (Abcam, Cambridge, UK), anti-cIAP1 (R&D Systems) or anti-cIAP2 (Epitomics, Burlingame, USA) antibodies followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz, Heidelberg, Germany). Next, membranes were developed by using an enhanced chemo luminescence detection system (ECL, Perkin Elmer, Waltham, USA) and Kodak films (Biomax®, Rochester, USA) for autoradiography. To confirm equal protein loading, membranes were subsequently reprobed with anti-ß-actin antibodies (Sigma Aldrich, Munich, Germany). Individual bands were quantified using the ImageJ 1.41 software package (National Institutes of Health, Bethesda, USA).

Clonogenic survival assay

The clonogenic colony formation assay was performed on single cell suspension as described previously [38]. Briefly, cells were

treated with artesunate for 24 h, plated into 6-well plates (BD Biosciences) and after an additional 4 h, cells were irradiated at room temperature as described above. After 11–14 days, colonies were stained with methylene-blue solution for 30 min and counted. Calculation of survival fractions (SF) was done using the equation SF = colonies counted/cells plated × (PE/100), taking into consideration the individual plating efficiency (PE). Survival variables α and β were fitted according to the linear quadratic equation ($SF = \exp[-\alpha \times D - \beta \times D^2]$ with D = dose using EXCEL® software (Microsoft, Unterschleißheim, Germany). Radiation enhancement ratios at 50% and 10% survival were calculated by transforming the above mentioned equation using α and β values of the individual survival curves.

Survivin–GFP expression construct and transfection

For the expression of survivin–GFP fusion protein, a human survivin cDNA was amplified with specific primers (Surv-fw: 5′-gg-ggtacc-ggcggc-ATGGGTGCCCCGACGTTGC-3′; Surv-rev: 5′-cg-ggatcc-cg-ATCCATGGCAGCCAGCTGCTC-3′), flanked with *Kpn*I and *Bam*HI restriction sites, from an expression plasmid kindly provided by R.H. Stauber (University Hospital of Mainz, Mainz, Germany). Subsequently, PCR fragments digested with *Kpn*I and *Bam*HI (New England Biolabs, Frankfurt am Main, Germany) were inserted into *Kpn*I/*Bam*HI sites of pEGFP-N1 expression vector (Clontech, Saint-Germain-en-Laye, France). LN229 cells were stably transfected with pEGFP-N1 (GFP) or pEGFP-survivin (survivin–GFP) expression constructs using Roti-Fect PLUS transfection reagent (Carl Roth, Karlsruhe, Germany) according to manufacturer's instructions. After selection with G418 (PAA), clones were isolated and expression of GFP or survivin–GFP was verified by fluorescence microscopy and Western blotting.

Statistical analysis

Experimental data are presented as mean ± standard deviations from at least three or more independent experiments. Levels of significance were calculated using Student's *t*-test (EXCEL® program).

Results

To first evaluate a cytotoxic effect of artesunate on glioma cells, we treated LN229 and U87MG cells with varying concentrations of the drug and measured cell numbers 48 h after treatment by a colorimetric MTT-assay. The results displayed in Supplemental Fig. 1 revealed that as compared to mock or DMSO treated controls, artesunate reduced cell numbers in a dose-dependent manner with a significant reduction at doses above 8 μg/ml (LN229) and 16 μg/ml (U87MG), resulting in an IC_{50} of 12.1 and 21.34 μg/ml, respectively.

To explore an effect of artesunate (24 h) and irradiation on cell numbers and apoptosis at 48 h following combined modality treatment we performed MTT and caspase 3/7 assays and determined the fraction of cells in a subG1 phase. As depicted in Fig. 1A, pre-incubation with 4 μg/ml artesunate for 24 h and irradiation with a dose of 2 and 8 Gy reveal a significantly decreased number of LN229 cells ($p < 0.001$) and U87MG cells (8 Gy, $p < 0.01$) as compared to mock-treated controls. In parallel, caspase 3/7 activity increased after combined artesunate and irradiation treatment (Fig. 1B) in an additive manner with significant values ($p < 0.01$) at 2 and 8 Gy for LN229 and at 8 Gy for U87MG. An elevated number of apoptotic cells was further confirmed by an increased detection of cells in a subG1 fraction by cytofluorometric analysis (Fig. 1C). Notably, the cell lines differ in their responsiveness toward artesnuate/irradiation treatment by apoptosis induction with a more pronounced effect in the line LN229.

Cell cycle analyses performed at 48 h after irradiation of LN229 and U87MG cells pre-treated with 4 μg/ml artesunate for 24 h revealed an increased percentage of cells in the G2/M-phase of the cell cycle in artesunate- and irradiation-treated glioma cells, indicating that a larger amount of cells were blocked in a more radiosensitive phase of the cell cycle (Table. 1). As compared to a marginal increase in the line U87MG, induction of a G2/M arrest was significant as compared to mock-treated cells ($p < 0.005$) in the line LN229. Notably, combined treatment with 16 μg/ml artesunate and irradiation also resulted in a significant reduction of cells in S and G1 phases, probably due to increased cell death by apoptosis.

Following knock down of the IAP Survivin, a sensitization of tumor cells to ionizing irradiation has been described, which is mediated by increased apoptosis and caspase-independent mechanisms like increased G2/M growth arrest and an impaired DNA damage response [12,37,40]. Thus, we next asked, whether an increase of apoptotic cell death and increased fraction of cells in the G2/M phase of the cell cycle following artesunate/irradiation treatment may be modulated by impairment of members of the IAP family. Western blot analysis of total cellular extracts generated at 0–48 h after artesunate incubation indicated a markedly reduced survivin protein expression in LN229 cells at 24–48 h after treatment (Fig. 2A) and artesunate ranging from 2 to 32 μg/ml (LN229 and U87MG, Fig. 2B and C). Densitometric analysis further yielded a 60–90% reduction of survivin protein expression at 36 and 48 h after treatment and incubation with 4–32 μg/ml artesunate. On the contrary, the expression of the IAPs X-linked inhibitor of apoptosis protein (XIAP), cellular IAP1 (cIAP1) and cellular IAP2 (cIAP2) was not affected by artesunate treatment, indicating a selective down-regulation of survivin (Fig. 2A).

Recent data indicate an impact of artesunate on DNA damage induction and repair by homologous recombination (HR) and non homologous end-joining (NHEJ) [31]. To further explore putative underlying mechanisms, we next analyzed whether artesunate treatment may interfere with irradiation-induced double-strand break repair. For this purpose, LN229 and U87MG cells were treated with artesunate and irradiation, and subjected to γ-H2AX immunofluorescence analysis at early (0, 20, 40, 60 min) and later time points (2, 6, 12, 24 h). As compared to mock or DMSO-control treated cells we detected significant (LN229: $p < 0.003$; U87MG: $p < 0.02$) increased γ-H2AX foci per nucleus in artesunate-treated cells with maximum values at 60 min after irradiation (Fig. 3B) and persisting significantly ($p < 0.05$) elevated levels at 24 h after combined modality treatment compared to sole irradiated and artesunate treated and non-irradiated (baseline, 0 h) controls (Fig 3A).

To further establish a correlation of artesunate treatment and radiation response, clonogenic survival assays were performed. Pre-incubation of LN229 and U87MG cells with 4 and 8 μg/ml artesunate significantly reduced basic clonogenic survival in comparison to mock-treated controls (Fig. 4A). Moreover, pre-treatment with both artesunate concentrations significantly radiosensitized the cell lines (Fig. 4), resulting in a calculated radiation-induced cytotoxicity enhancement factor of 1.65 (LD_{50}) for LN229 and 1.30 (LD_{50}) for U87MG after treatment with 8 μg/ml artesunate (Table 2). By contrast, clonogenic survival assays performed after pre incubation for 24 h with 8 μg/ml artesunate in LN229 cells stably over expressing a survivin–GFP fusion protein (Fig. 5A) reversed artesunate-induced radiosensitization as compared to GFP-control-transfected LN229 cells (Fig. 5B). Moreover, as compared to parental cells, a significantly decreased caspase3/7 activity was observed in survivin–GFP transfected LN229 cells following treatment with artesunate and irradiation with a dose of 2 and 8 Gy, respectively (Fig. 5C).

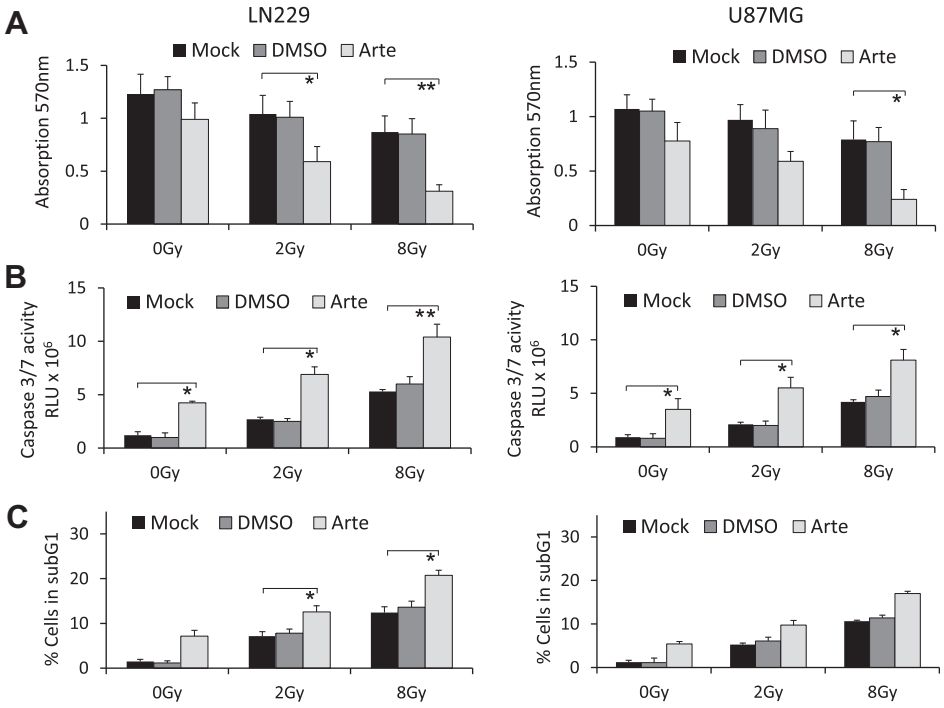

Fig. 1. (A) LN229 and U87MG glioblastoma cells were treated with artesunate (4 μg/ml) for 24 h and subsequently irradiated with a dose of 2 or 8 Gy. Forty-eight hours after treatment cells were subjected to a colorimetric MTT-assay. (B) Analysis of caspase 3/7 activity at 48 h following treatment with artesunate (24 h) and subsequent irradiation. (C) Analysis of apoptotic cells as determined by flow cytometry of the fraction of cells in a sub-G1 phase. Data are displayed as mean ± SD from three experiments. Asterisks indicate significant differences ($^{*}p < 0.03$, $^{**}p < 0.001$) as compared to mock-treated controls.

Table 1
Increased percentage of cells in the G2/M-phase in artesunate and irradiation treated glioma cells. At 24 h after treatment with artesunate (4 and 16 μg/ml) and irradiation (2 Gy) LN229 and U87MG glioma cells were labeled with propidium iodide (PI) and flow cytometry was used to measure DNA content. Data are displayed as the mean ± SD from at least six to seven independent experiments (individual *p* values versus mock-treated cells are depicted in the table).

Treatment	LN229			U87MG		
	% Cells in G1	% Cells in S	% Cells in G2/M	% Cells in G1	% Cells in S	% Cells in G2/M
Mock-treated	50.5 ± 2.9	19.6 ± 1.6	14.7 ± 1.8	36.6 ± 1.4	15.7 ± 3.6	20.4 ± 0.6
+2 Gy	46.6 ± 2.1	18.5 ± 2.4	17.1 ± 1.1	36.3 ± 1.2	10.9 ± 1.4	22.3 ± 0.7
Arte (4 μg/ml)	44.9 ± 3.8	16.5 ± 2.1	18.5 ± 2.0	36.1 ± 1.5	8.8 ± 1.3	23.0 ± 0.4
Arte (4 μg/ml) + 2 Gy	44.7 ± 2.7	14.4 ± 1.5	22.9 ± 1.4 *p < 0.005*	38.8 ± 0.7	7.9 ± 1.4	24.1 ± 0.5
Arte (16 μg/ml) + 2 Gy	30.7 ± 1.9 *p < 0.002*	8.6 ± 1.1 *p < 0.002*	27.1 ± 1.7 *p < 0.002*	39.4 ± 1.8	5.3 ± 1.8 *p < 0.05*	25.3 ± 0.8 *p < 0.05*

Discussion

In the present study, we show that artesunate and X-irradiation dose-dependently suppress clonogenic survival in both LN229 and U87MG glioma cells (Fig. 4). Moreover, we report that combined modality treatment induces caspase-dependent apoptosis (Fig. 1), increases G2/M cell cycle arrest (Table 1), modulates DNA-damage response (Fig. 3) and attenuates expression of the inhibitor of apoptosis protein survivin (Fig. 2). These results suggest that artesunate displays a promising candidate as an adjuvant drug to radiation therapy in glioma cancer that is in line with an established radiosensitizing effect reported by dihydroartemisinin in U373 glioblastoma cells [28].

Cellular response to ionizing radiation has been shown to be mediated by the production of radicals and reactive oxygen species (ROS) [23,34] that in turn target a variety of macromolecules including DNA and proteins. The active moiety of artesunate is an endoperoxide bridge [45] which is cleaved in a ferrous ion dependent manner to result in the formation of reactive oxygen species (ROS), and carbon-centered radicals [10,32]. Although we did not focus on radical production in our present investigation, it has convincingly been shown that ROS production contributes to the cytotoxic effect of artesunate in tumor cells [8,18,19,28]. Kim et al. reported that a radiosensitization achieved by treatment of U373 cells with dihydroartemisinin was blocked significantly by the free radical scavenger *N*-acetyl cysteine (NAC) indicating an association between radiation response and artemisinin induced ROS generation [28]. More recently, Berdelle et al. [8] further confirmed that artesunate treatment results in oxidative DNA damage, resulting in an increased number of DNA double stand breaks (DSB) as proven by phosphohistone γ-H2AX and 53BP1 foci in LN229 glioblastoma cells, which is in line with our investigations (Fig. 3). In addition, a ROS-dependent induction of apoptosis by a lysosome-dependent mitochondrial outer membrane permeabilization or oxidative DNA damage has been reported to contribute to artesunate's anti cancer effect [8,16,18,24]. An involvement of apoptosis is further strengthened by our present results, indicating that artesunate increases caspase 3/7 activity and the fraction of cells in a subG1 status that is augmented by combined treatment with ionizing radiation (Fig. 1).

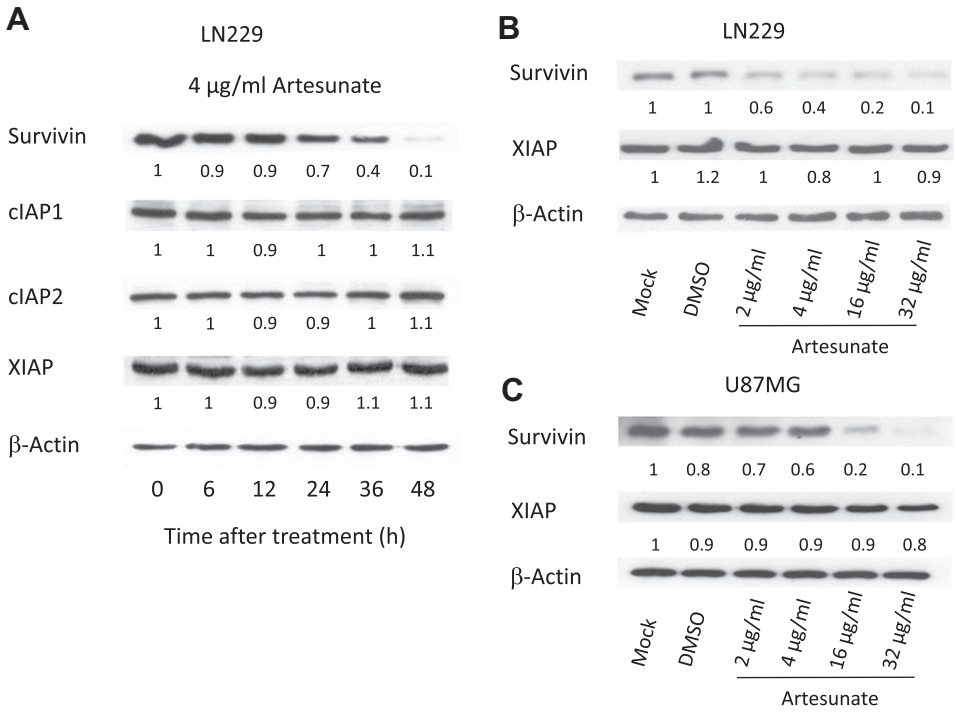

Fig. 2. Down regulation of survivin protein in LN229 and U87MG glioblastoma cells treated by artesunate. Western immunoblots from total cellular proteins extracted at the indicated time points (A) or concentrations (B) after treatment with artesunate for 24 h using antibodies against survivin, XIAP, cIAP1, cIAP2 and actin for loading control. Data are displayed as one representative out of three independent experiments. Numbers indicate reduction of protein expression as compared to β-actin control as determined by densitometric analysis using the ImageJ software package.

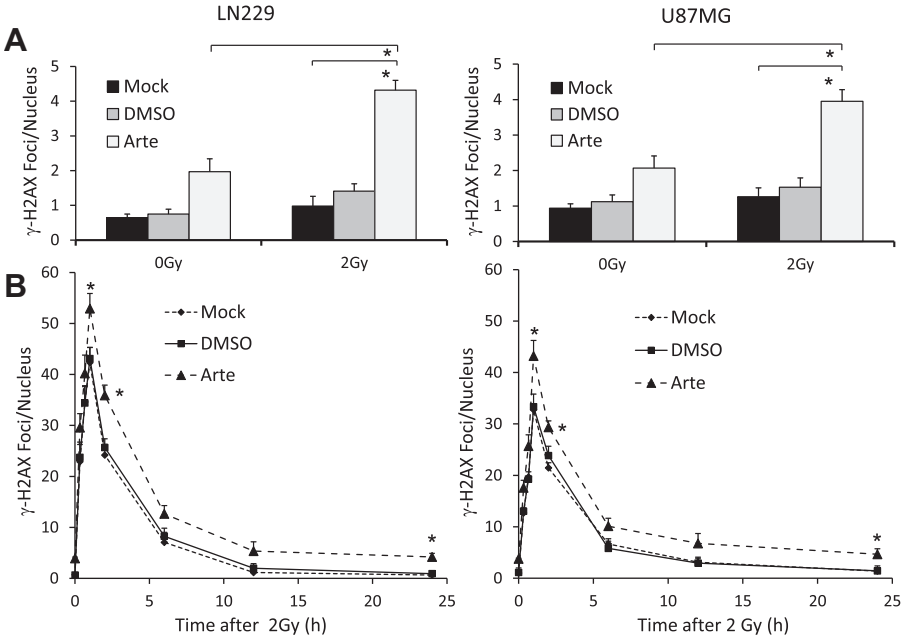

Fig. 3. Residual damage at 24 h (A) and (B) kinetics of serine 139 phosphorylated histone γ-H2AX foci detection per nucleus after combined modality treatment. LN229 and U87MG glioma cells were cultured on 8-well slides, treated with 4 µg/ml artesunate for 24 h and irradiated with a dose of 2 Gy to assure a discrimination of individual nuclear foci in immuno-fluorescence staining using γ-H2AX primary, Alexa-594 secondary antibody and DAPI for nuclear counterstaining. Data are given as mean ± SD from three repeated experiments ($^{*}p < 0.05$ versus mock-treated cells). For each data point 150–200 nuclei were evaluated.

In search of genes involved in the cytotoxic effect of artemisinin, microarray expression analysis revealed a variety of factors that affect cellular response to the drug including apoptosis regulating genes [4,15,20]. Here we further propose that a selective down regulation of the multifunctional protein survivin contributes to artesunate's anti-cancer effects. During the last decade, survivin deserves growing attention due to its universal over expression in malignant cells, its prognostic relevance and its prominent role in the regulation of a variety of cellular networks, including apoptosis, tumor cell proliferation and adaption to an unfavorable environment [3]. Due to these unique properties, the protein has been proposed as an attractive molecular target for

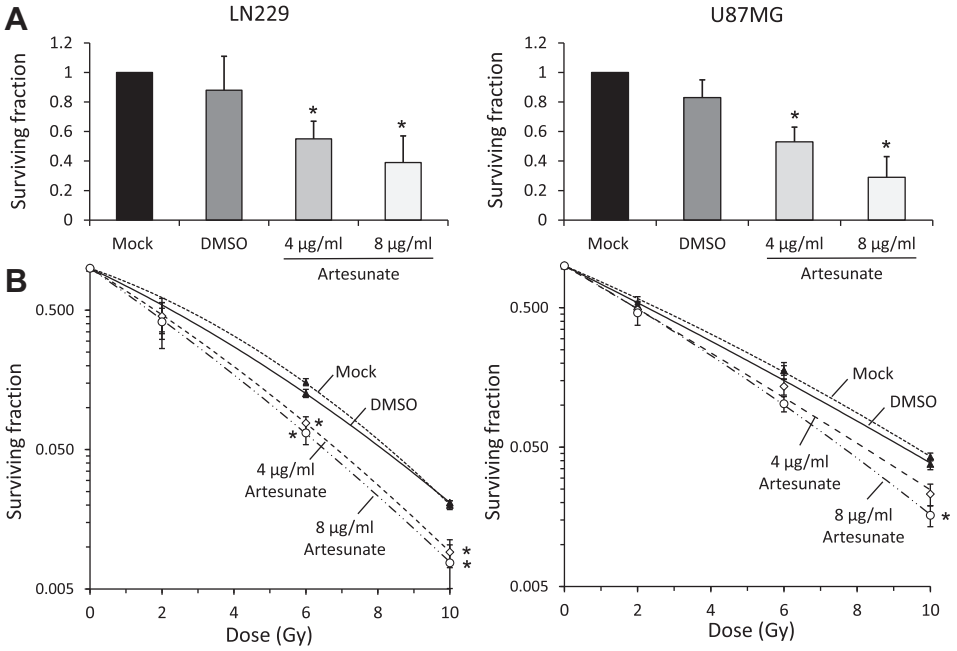

Fig. 4. Clonogenic survival of LN229 and U87MG cells pretreated for 24 h with artesunate at a concentration of 4 and 8 µg/ml and irradiated with the doses indicated. Mock or DMSO treated cells served as a control. (A) After 12–14 days, colonies greater than 50 cells were counted and basal clonogenic survival was determined. (B) Survival curves with surviving fractions (SF) normalized to the plating efficiency were fitted according to the linear quadratic equation: $SF = \exp[-\alpha \times D - \beta \times D^2]$ with D = dose. Data are displayed as the mean ± SD from three independent experiments ($^*p < 0.04$, versus mock- or DMSO treated cells).

Table 2
Radiation response variables of LN229 and U87MG cells after combined modality treatment with artesunate (4 and 8 µg/ml). Radiation-induced cytotoxicity enhancement factors at 50% survival (LD_{50}) and 10% survival (LD_{10}) were calculated by transforming the linear quadratic equation using α and β values of the individual survival curves.

Cell line treatment	Plating efficiency (%)	α (Gy^{-1})	β (Gy^{-2})	LD_{50} (Gy)	Radiation enhancement ratio	LD_{10} (Gy)	Radiation enhancement ratio
LN229							
Mock-treated	37.2	0.2061	0.0184	2.71		6.91	
Arte (4 µg/ml)	18.6	0.3641	0.0104	1.81	1.50	5.47	1.26
Arte (8 µg/ml)	13.2	0.4105	0.0075	1.64	1.65	5.13	1.35
U87MG							
Mock-treated	25.3	0.2595	0.0055	2.53		7.63	
Arte (4 µg/ml)	10.0	0.3521	0.0017	1.95	1.29	6.35	1.20
Arte (8 µg/ml)	7.8	0.3426	0.0068	1.95	1.30	6.00	1.27

anticancer therapies. Indeed, several preclinical studies have demonstrated that targeting survivin expression and function by RNA interference, antisense oligonucleotides (ASO) and small molecule repressors sensitized tumor cells toward irradiation and reduces tumor growth potential [27,40]. Notably, it now became increasingly clear that the role of survivin in cellular response to anticancer treatment far exceeds a simple inhibition of apoptotic cell death but also involves regulation of the cell cycle and DNA-damage response [2,40]. This functionality may well fit to the nodes of action exerted by treatment of cancer cells with artesunate.

In order to further explore underlying mechanisms of artesunate's cytotoxic effect in combination with ionizing radiation, recent reports indicate, that the drug induces the formation of DNA-DSBs that in turn trigger a DNA damage response (DDR) as proven by phosphorylation of Ataxia telangiectasia mutated (ATM), Ataxia telangiectasia and Rad3 related (ATR), checkpoint kinase 1 (Chk1) and Chk2 [8]. Moreover, knockdown of Rad51 by siRNA and inactivation of DNA-dependent protein kinase (DNA-PK) strongly sensitized LN229 glioma cells to artesunate treatment indicating that both homologous recombination (HR) and non-homologous end joining (NHEJ) may be involved in the repair of artesunate-induced DSB [8,31]. From these data, however, it is not clear whether artesunate directly or indirectly interferes with DNA damage response. We have recently shown that a nuclear accumulation of survivin is associated with DNA damage repair in both colorectal and glioblastoma cells [11,37] by a direct interrelationship with members of the NHEJ repair machinery (e.g. H2AX, MDC1 and DNA-PKcs). A survivin knockdown by siRNA or ASO resulted in significant increased γ-H2AX foci detection at 40–60 min and persisting elevated levels at 12–24 h after irradiation [38,39] similar to the data in the present study (Fig. 3). It is therefore tempting to speculate, that at least in part, radiosensitization achieved by artesunate treatment in LN229 and U87MG glioma cells is mediated by a survivin attenuation. To further investigate a causal impact of survivin in artesunate sensitivity, a LN229 clone stably over expressing a survivin–GFP fusion protein was established and treated with or without artesunate and irradiation (Fig. 5). As a survivin over expression in artesunate treated LN229 significantly partly restored an apoptosis resistant phenotype (Fig. 5C) and reversed artesunate-mediated radiosensitization (Fig 5B), our data favor a direct and causal role of survivin in artesunate cytotoxic activity.

An involvement of survivin in artesunate-mediated anti-cancer effects is further supported by a report from Xu et al., indicating that survivin expression in human osteosarcoma cells was diminished after artesunate treatment in a dose-dependent manner,

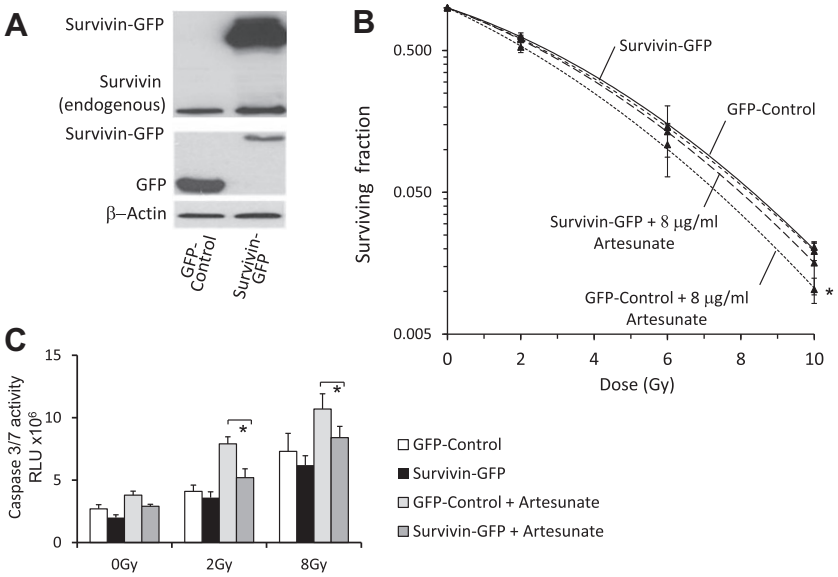

Fig. 5. LN229 cells were transfected with pEGFP-N1 (GFP-control) or pEGFP-survivin (survivin–GFP) expression plasmids and stable clones were established by G418 selection. Over expression of GFP or survivin-GFP fusion protein was verified by Western immunoblotting (A). Clonogenic survival of GFP-control and survivin GFP-expressing LN229 cells pre-treated for 24 h with artesunate at a concentration of 8 μg/ml and irradiated with the doses indicated. Mock artesunate treated GFP expressing LN229 served as a control. Data are displayed as the mean ± SD from three independent experiments ($^{*}p < 0.05$). (C) Analysis of caspase 3/7 activity at 48 h following pre-treatment with artesunate (24 h) and subsequent irradiation in LN229 survivin–GFP and GFP-control cells. Data are displayed as mean ± SD from three experiments ($^{*}p < 0.05$ as compared to GFP-controls).

both *in vitro* and in a xenograft tumor model [46]. Moreover, upon treatment with artesunate, TNF-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity in HeLa cells was enhanced by suppressing pro-survival proteins, such as survivin and XIAP in a nuclear factor kappa B (NF-κB) and PI3 K/Akt signaling pathway-dependent manner [43]. These data are in part contradictory to our results, as we do not see a down regulation of XIAP in LN229 and U87MG cells. However, they confirm the involvement of anti-apoptotic molecules in artesunate's anti-tumor activity and foster proceeding experiments on the underlying mechanisms.

As reported for a variety of tumor cell lines [17,20,28], our investigations further revealed a different response of glioma cells to artesunate with a more pronounced effect in LN229 cells. In line with this, the expression of genes that significantly correlate to the IC_{50} values for artesunate was evaluated in a panel of 55 cell lines from the US National Cancer Institute (NCI) [17,20]. By using pharmacogenetic and molecular pharmacological approaches a variety of candidate genes were identified. Among these factors, apoptosis regulating genes including Bcl2, and NF-κB were reported to contribute to artesunate resistance [15]. In line with that, a more recent report further demonstrated that an artesunate resistant phenotype in MDA-MB-231 breast cancer cells is associated with the up regulation of the transcription factor NF-κB and activating protein (AP-1) that in turn increase the expression of anti-apoptotic Bcl-2 and reduce the expression of pro-apoptotic bax [6]. Although a role of NF-κB in the regulation of survivin is well established [5,44], whether a differential regulation of NF-κB and AP-1 contributes to therapeutic effect of artesunate if combined with ionizing radiation, requires ongoing investigations.

In conclusion, our data support the view that artesunate sensitizes glioma cells to ionizing irradiation by multiple mechanisms including attenuation of survivin expression, increased apoptosis, and a hampered DNA damage repair. Although a radiosensitizing effect of fractionated irradiation still has to be confirmed in proceeding preclinical and especially in continuative animal models, these data further suggest that combined modality treatment including artesunate may increase the therapeutic effectiveness of radiation therapy in glioblastoma.

Conflict of interest statement

There are no actual or potential conflicts of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2012.03.018.

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Survivin-miRNA-loaded nanoparticles as auxiliary tools for radiation therapy: preparation, characterisation, drug release, cytotoxicity and therapeutic effect on colorectal cancer cells

Sebastian Gaca[1], Sebastian Reichert[2], Claus Rödel[2], Franz Rödel[2] and Jörg Kreuter[1]

[1]*Institute of Pharmaceutical Technology, Goethe University, Frankfurt am Main, Germany, and*
[2]*Department of Radiotherapy and Oncology, Goethe University, Frankfurt am Main, Germany*

Abstract

One of the main challenges in radiation oncology is to overcome the resistance of cancer cells against treatment by molecular targeted approaches. Among the most promising targets is the inhibitor of apoptosis protein survivin, known to be associated with increased tumour aggressiveness and therapy resistance. The objective of this study was the development of a human serum albumin-based nanoparticulate carrier system for plasmid-mediated RNA interference (miRNA) and the investigation of its *in vitro* efficacy on survivin knockdown and cellular toxicity in SW480 colorectal cancer cells. The results demonstrate a robust nanoparticulate system of a size around 220 nm with a plasmid incorporation efficacy of about 90%. Moreover, treatment of carcinoma cells with survivin-miRNA nanoparticles resulted in reduction of survivin expression by 50% and increased cytotoxicity if combined with ionising irradiation. These nanoparticles comprise a promising option to enhance the response of carcinoma cells to therapy with ionising irradiation.

Keywords: nanoparticles, survivin, miRNA, ionising radiation

Introduction

Nanoparticles (NPs) as drug carrier systems were first introduced by Speiser and co-workers in the late 1960s. Since then, the interest in NPs in many fields of research has grown exponentially. Following Speiser's work, Marty and Oppenheim have developed human serum albumin (HSA)-NP employing the method of desolvation (Kreuter, 2007). HSA as the basic component for NPs production shows a number of advantages, such as a good tolerance in humans and a variety for surface modification using its functional groups (Weber et al., 2000; Zensi et al., 2009). Promising results were obtained with HSA-NP used for the delivery of plasmid DNA to cells, as they showed a superior therapeutic effect on cancer cells (Rhaese et al., 2003; Steinhauser et al., 2009) These NPs were able to overcome restricting problems of gene therapy with naked DNA by preventing degradation by nucleases, and increasing cellular uptake which were claimed to be part of the main problems of vector based gene therapy (Nishikawa et al., 2005). NPs are capable to target cancer cells via the enhanced permeation and retention effect (Maeda, 2001; Greish, 2010) as the NPs are able to enter the tumour tissue through leaky vessels and are retained because of reduced lymphatic drainage.

Alternative carriers such as viral vectors (Gardlik et al., 2005) show a good DNA transfection efficiency, but are afflicted by high costs or increased toxicity (Boulaiz et al., 2005). Moreover, a multitude of NP-based trials in gene therapy operate with cationic polymers such as polyethylenimine yielding a positive surface charge in order to attain a lysosomal escape (Zhang et al., 2008). These cationic polymers, however, again appear to increase the toxicity of the preparation (Godbey et al., 1999; Hunter, 2006). Furthermore, some groups showed that gene therapy with NPs may lead to significant therapeutic

Address for correspondence: Jörg Kreuter, Institute of Pharmaceutical Technology, Goethe University, Max-von-Laue-Strasse 9, D-60438 Frankfurt am Main, Germany. Tel: +49 69 798 29682. Fax: +49 69 798 29694. E-mail: Kreuter@em.uni-frankfurt.de

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improvements which are comparable to polycationic vectors without showing the risks associated with the polycations (Mo et al., 2007; Steinhauser et al., 2009).

Due to its universal overexpression in human cancerous tissues and its prominent nodal role in disparate networks of cellular division, intracellular signalling and apoptosis, the inhibitor of apoptosis protein survivin deserves growing interest as a suitable target of a molecular targeted tumour therapy (Altieri, 2008). In line with that, several preclinical studies have demonstrated that targeting survivin expression by the use of RNA interference, antisense-oligonucleotides and small molecule repressors sensitised tumour cells towards chemotherapeutic drugs and irradiation and reduced tumour growth potential (Reichert et al., 2011; Rödel et al., 2011). Moreover, NPs loaded with survivin siRNA have already proven efficacy in enhanced chemosensitivity of MCF-7 breast cancer cells to adriamycin *in vivo* (Yang et al., 2010), but to the best of our knowledge, a radiosensitising effect of survivin-miRNA plasmid-loaded NPs has yet not been reported. Thus, the purpose of this study was to develop and characterise a survivin-miRNA plasmid-loaded HSA-NP system to efficiently knockdown survivin expression and to increase radiation responsiveness in SW480 colorectal adenocarcinoma cells.

Materials and methods

Chemicals and reagents

HSA (fraction V; purity 96–99%; 65 000 Da; batch 028K7550) was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Glutaraldehyde solution (25%) was purchased from VWR International GmbH (Darmstadt, Germany) and was used as an 8% solution, prepared with purified water. Proteinase K was obtained from Applichem GmbH (Darmstadt, Germany). Ethidium bromide and agarose were purchased from Roth GmbH (Karlsruhe, Germany).

Survivin and negative control miRNA plasmid

The human survivin-specific miRNA plasmid was generated by insertion of a double-stranded oligo (Eurofins MWG Operon, Ebersberg, Germany) containing the target pre-miRNA sequence (top strand: 5′-TGCTGAAGGATTTAGGCCACTGCCTTGTTTTGGCCACTGACTGACAAGGCAGTCCTAAATCCTT-3′; bottom strand: 5′-CCTGAAGGATTTAGGACTGCCTTGTCAGTCAGTGGCCAAAACAAGGCAGTGGCCTAAATCCTTC-3′) in the pcDNA6.2-GW/EmGFP-miR expression vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The reverse complement of the 21-nucleotide sense target sequence (mature miRNA sequence) is as follows: 5′-AAGGATTTAGGCCACTGCCTT-3′ and corresponds to the human survivin-specific siRNA sequence 5′-GGCAGUGGCCUAAAUCCUUtt-3′ (sense; siRNA ID #121296; Applied Biosystems, Darmstadt, Germany). To increase knockdown efficiency, the survivin-specific pre-miRNA sequence including 5′ and 3′ miR flanking regions was duplicated by miRNA chaining according to the manufacturer's protocol. For controls, the non-specific pre-miRNA sequence (Top Strand: 5′ TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′) of the pcDNA^{TM}6.2-GW/EmGFP-miR-neg control plasmid (Invitrogen, Karlsruhe, Germany) including 5′ and 3′ miR flanking regions was duplicated as described above.

Cell culture

Human colorectal adenocarcinoma cells SW480 were purchased from the American Type Culture Collection (LGC-Promochem, Wiesbaden, Germany) and cultivated in Dulbecco's Modified Eagle's Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% bovine serum (FBS Superior), and 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all supplements from Biochrom, Berlin, Germany) at 37°C, 5% CO_2 and 95% humidity.

Preparation of plasmid-HSA-NP

Plasmid-HSA-NP preparation was performed by modification of a previously described desolvation method (Steinhauser et al., 2009). In brief, HSA (40 mg) was dissolved in 1 mL of 10 mM sodium chloride solution. This solution was adjusted to a pH between 6 and 8. Afterwards, the albumin solution was filtered through a 0.2 μm sterile filtration unit (Whatman GmbH, Dassel, Germany). The plasmid-solution was diluted to 500 μL with purified water to concentrations ranging between 0 and 300 μg/mL and mixed with 500 μL of the previously prepared albumin solution for 15 min using a magnetic stirrer at 550 rpm at room temperature. Then, NPs were formed by the addition of 2.7 mL of ethanol (96%) at a flow rate of 1 mL/min using a peristaltic pump (Ismatec IPN, Glattbrugg, Switzerland). After this desolvation step, 11.8 μL of 8% glutaraldehyde solution was added dropwise, in order to form stable NPs by crosslinking the amino-groups of the HSA. The NPs were next stirred at 550 rpm at 22°C over night or at least 12 h to achieve a sufficient crosslinking. After this process, the NPs were purified by centrifugation ($3 \times 16\,000 \times g$, 8 min) and redispersion of the NP pellet in purified water under sonication (Sonorex Super RK102H, Bandelin, Berlin, Germany) and vortexing.

Characterisation of plasmid-HSA-NP: particle size, polydispersity, surface charge and particle yield

For the photon correlation spectroscopic (PCS) determination of the mean particle size, the polydispersity index of the size distribution and the zeta potential, a Malvern Zetasizer 3000HSA (Malvern Instruments Ltd, Malvern, UK) at a scattering angle of 90°, at room temperature was used. The sample was diluted 1:400 times with purified water prior to measurement. The zeta potential

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measurements were carried out with dip-cells fitting to the Zetasizer. The efficacy of the desolvation process was determined by microgravimetry (Kufleitner et al., 2010). For this purpose, 50 μL of the NP suspension concentrated to a volume of 1 mL was filled in a pre-weighed aluminium weighing dish (VWR, Darmstadt, Germany). After 2 h of desiccation at 80°C, the pans were allowed to cool down in an excicator for 0.5 h. The weighing dishes were weighed again with a micro-balance (Sartorius Supermicro, Göttingen, Germany). From the difference of the two measurements, the particle yield was calculated.

Determination of plasmid amount loaded into the NPs

For the determination of the plasmid amount that was incorporated into the NPs, agarose gel electrophoresis was used. Before electrophoresis, NPs (2 mg) were digested with 2 μg/mL proteinase K in phosphate buffer pH 7.5 (Langer et al., 2008). These samples were incubated at 37°C under a constant shaking at 250 rpm (Eppendorf Thermomixer 5436, Eppendorf AG, Hamburg, Germany), and plasmids were purified by phenol/chloroform extraction. The plasmid concentration was determined in the digested NPs.

Release and stability analysis

To test the stability of plasmid-HSA-NPs, particles were prepared as described in ''Preparation of plasmid-HSA-NP'' section at a pH of 6.5 and the plasmid was used at a concentration of 100 μg/500 μL. The produced NPs were divided into aliquots of 1 mL with a particle content of 5 mg. One-half of the particles was stored at 4°C and the other half at 22°C. At the start and 1, 2, 4, 8, 10, 16 and 24 weeks later, one aliquot of each group was analysed for particle size, polydispersity index and zeta potential. The particle suspensions were centrifuged, and the supernatants tested for released plasmids. In the beginning and at the end of this study, one aliquot of the NPs was digested, and the incorporated amount of plasmid was analysed as described above.

The determination of the released plasmids from the NPs was performed using a method for PLGA-NP (Basarkar et al., 2007) which was optimised for the HSA system. In brief, an aliquot of 10 mg of NPs was separated, centrifuged and redispersed in 1 mL phosphate-buffered saline (PBS). These samples were shaken at 37°C for 14 days at 550 rpm on a thermomixer. After defined times, samples were centrifuged ($16\,000 \times g$; 8 min) and the supernatant was stored at −20°C. The pellet was redispersed in 1 mL fresh PBS under sonication and vortexing, and further shaken. The sampled supernatants as well as the remaining pellet after digestion were then analysed for plasmid content.

DNAse stability of loaded DNA

Plasmids incorporated into NPs are supposedly protected against external influences, especially nucleases DNAse 1 and DNAse 2. For this purpose, the influence of DNAse 1 was measured using the method of Niu et al. (2009). Briefly, 5 mg of NPs were incubated at 37°C for 30 min with DNAse 1 at three different concentrations (0.25, 0.5 and 1.0 U/μg DNA) in Mg^{2+} digestion buffer (50 mM, Tris–HCl, pH 7.6 and 10 mM $MgCl_2$). Naked plasmids with the same concentration served as a control. After incubation, DNAse 1 was inhibited with EDTA solution (50 mMol), the NPs were digested with proteinase K and plasmids were extracted. The resulting plasmid solutions were separated by gel electrophoresis in a 1.5% (w/v) agarose gel in TAE buffer (0.04 M Tris–HCl, 0.01 M EDTA, 0.02 M HAC and pH 8.5) and the plasmid conformation as well as the amount were analysed with ethidium bromide-staining on the agarose gel.

Scanning electron microscope pictures

The NPs were additionally investigated using a field emission electron microscope (Hitachi S-4500, Tokio, Japan). For this purpose, 10 μL of freshly prepared NPs diluted in ultrapure water were placed on an aluminium sample plate and allowed to dry at 22°C for 24 h. Then, the dryed NPs were sputtered with gold for 55 s under inert gas (Agar sputter coater, Agar Scientific, Stansted, England). Pictures were taken and analysed using a photosystem software (Point Electronic, Halle, Germany).

In vitro *knockdown of Survivin by HSA-NP*

To investigate the *in vitro* efficacy of the survivin-miRNA plasmid incorporated NPs, SW480 cells were seeded in six well plates at a cell count of 2×10^5 per well to reach a confluence of 50–70%. NPs with different amounts of incorporated plasmids (0, 10, 50, 100, 200 and 300 μg) or scrambled plasmid controls (200 μg/mL NPs) were diluted with cell-culture media without antibiotics. Subsequently, the cells were washed with PBS, and freshly prepared NP suspensions were added, incubated for 24 h and replaced by culture medium. After 96 h, cells were harvested and cell lysates were prepared in RIPA-buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na-Deoxycholate, 1% Triton X-100 or NP-40). Protein concentrations were determined using a microBCA-test (Thermo-Fisher, Waltham, MA) and 50 μg of total protein was loaded on a 12% SDS-polyacrylamide gel. The separation took place in a Biorad Mini-Protean Tetra System (Bio-Rad, München, Germany). Subsequently, the proteins were transferred to a nitrocellulose membrane (GE Healthcare, München, Germany) and probed either with anti-survivin antibodies (Rabbit anti-Human Survivin Affinity Purified Polyclonal Ab IgG, #AF886, R&D Systems, Heidelberg, Germany) or anti-β-actin (Monoclonal

antibody; A5441–clone AC-15; Sigma–Aldrich, Munich, Germany) as a loading control. Horseradish-peroxidase coupled appropriate secondary antibodies (Santa Cruz, Heidelberg, Germany) and a chemiluminescence detection system (ECL, Thermo-Fisher, Waltham, MA) were used for development, and luminescence X-ray films (GE Healthcare, München, Germany) and Optimax Type-RX (MS-L GmbH; 69234 Dielheim, Germany) apparatus for detection.

Colorimetric MTT assay and irradiation procedure

The cytotoxicity of the NPs and of the NPs in combination with radiation was determined using the MTT-assay. In brief, SW480 cells were cultivated in 96-well plates for 24 h at a density of 2500 cells/well in a volume of 100 μL in full medium. After that, the medium was replaced with the same volume of media without antibiotics. NPs with different amounts of incorporated survivin-miRNA plasmids or scrambled plasmids in concentrations ranging from 0 to 300 μg were diluted with antibiotic free cell-culture media to reach final concentrations of: 400, 200, 100, 50, 25, 12.5, 6.25 and 0 μg/mL, respectively. After 24 h of incubation, the media was collected and cells were washed with PBS. After washing, 200 μL of full medium was added to the cells. After 48 h, the cells were irradiated at room temperature with a single dose of 2 or 8 Gy using a linear accelerator (SL75/5, Elekta, Crawley, UK), with 6 MeV photons/100 cm focus-surface distance and a dose rate of 4 Gy/min. Another 48 h later, 20 μL of MTT reagent (Applichem, Darmstadt, Germany) dissolved at a concentration of 5 mg/mL in PBS was added to each well. After 4 h of incubation, 50 μL of 20% SDS 0.01 mol HCl solution was added to the cells and incubated over night. The amount of the violet formazan was determined spectrophotometrically at a wavelength of 570 nm in an ELISA reader (Victor Wallac Multilabel-reader, Perkin-Elmer, Waltham, MA). The cell viability (%) was calculated using the equation:

$$\text{viability } (\%) = \frac{OD_{570}(\text{samples})}{OD_{570}(\text{control})} \times 100$$

Statistical methods

All measurements were performed at least three times. Data are given as mean ± SD. To determine statistical differences between two groups ($p < 0.05$), paired and unpaired student *t*-tests were used (Sigmaplot11, Systat Software, Chicago, IL).

Results and discussion

HSA-NPs with incorporated plasmids were prepared at different pH values and increasing amount of plasmid DNA. These preparations were characterised with respect to size, polydispersity, zeta potential, particle yield and drug loading. An optimal preparation regarding high encapsulation yield and a narrow size distribution with a mean around 220 nm was selected, and further investigated concerning storage stability, DNAse protection and drug release.

Preparation of NPs

NPs showed a pH-dependent size distribution. The mean particle diameter and the zeta potential of unloaded particles (NP-E) as well as plasmid-loaded particles (NP-P) decreased with increasing pH (Figure 1; Table 1). This is to be expected for albumin NPs as the increased amount of negative charges as a result of the higher pH reduces agglomeration of albumin molecules during desolvation due to increased repulsive forces. The reduced agglomeration tendency in turn leads to smaller NP sizes (Langer et al., 2003). In the presence of incorporated plasmids, the size of the NPs decreased as compared to the empty particles. As mentioned above, a higher amount of negative charges decreased size of the NPs that was more pronounced in the presence of equally negatively charged DNA-plasmids.

Thus, the influence of the amount of incorporated DNA on particle size was analysed at pH 6.5 indicating that an increase in the DNA amount reduces both the NP size and the particle yield (Figure 1; Tables 1 and 2). Additionally, plasmid loading efficiency also displays a pH-dependency, with the most efficient incorporation at pH of 6.5 and 7.5 (Table 1). Dose kinetic experiments further revealed an incorporation efficiency between 10 and 50 μg of 90–100%. The efficiency decreased for 100 μg to 85% and 200 μg to about 80%. At 300 μg, only 65% of the plasmid was entrapped (Table 2).

Storage stability and release characteristics of plasmid-HSA-NP

Storage stability is a prerequisite for a future therapeutic application of NPs in clinical use. Therefore, changes in particle size, zeta potential and polydispersity were monitored over a period of 24 weeks at 4°C and at 22°C. As depicted in Figure 2, storage conditions did not change particle size significantly. Zeta potential fluctuated slightly over time around a mean of −35 mV, but always displayed sufficient surface charge to avoid a particle agglomeration (Langer et al., 2003). Polydispersity did also not change in a significant manner, with a narrow size distribution with a polydispersity index below 0.1 (Figure 2A) except for one outlier at week 16. By contrast, as compared to freshly prepared NPs, plasmid DNA content significantly decreased over time at both conditions, with a more pronounced effect after storage at room temperature (Figure 2B). Under both conditions, plasmids showed similar conformations and molecular weights as compared to native (stock) plasmids. This indicates that the DNA can be considered to be stable. Another important factor for

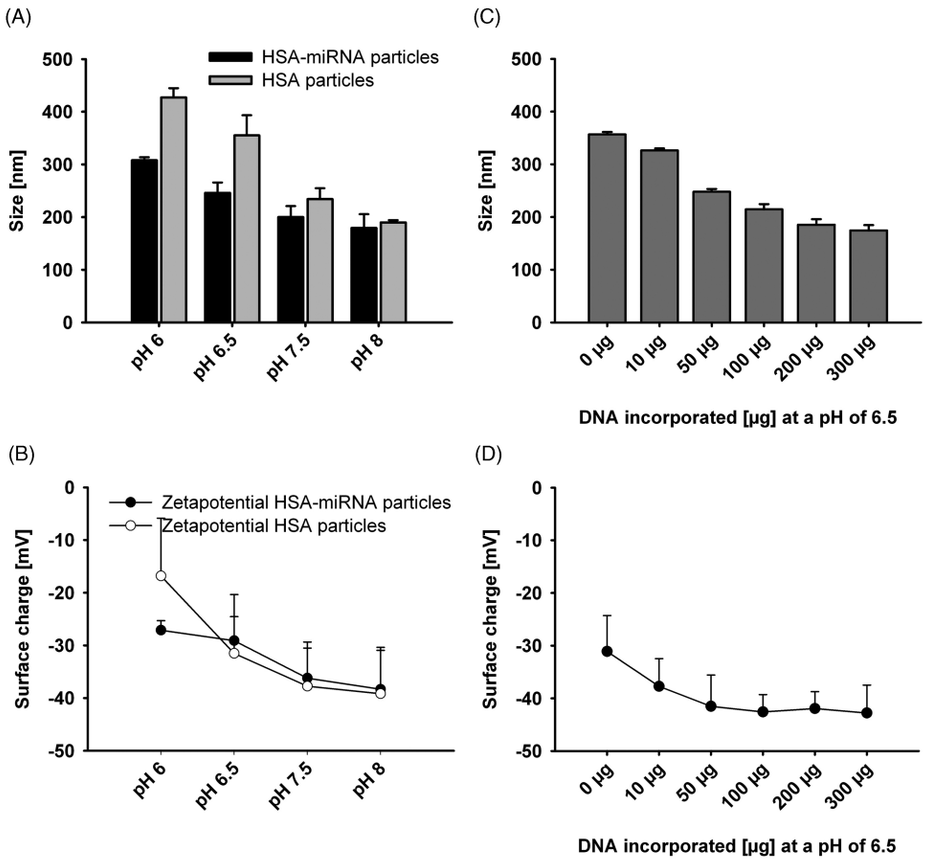

Figure 1. Influence of pH on size (A) and zeta potential (B) and of plasmid content at a pH of 6.5 on size (C) and zeta potential (D) of HSA-miRNA-plasmid NPs ($n = 3$; mean ± SD).

Table 1. Characterisation of NPs with different pH values at desolvation ($n = 3$).

NP formulation	Size (nm)	Polydispersity index	Zeta potential (mV)	Particle yield (mg)	Plasmid load (µg/mg)
NP-P pH 6	308 ± 6	0.06 ± 0.01	−27 ± 2	16.2 ± 0.2	4.4 ± 1.4
NP-P pH 6.5	246 ± 20	0.07 ± 0.02	−29 ± 9	16.8 ± 0.9	5.3 ± 0.5
NP-P pH 7.5	200 ± 21	0.04 ± 0.02	−36 ± 7	13.1 ± 0.5	7.3 ± 0.6
NP-P pH 8	179 ± 26	0.02 ± 0.01	−38 ± 7	4.4 ± 2.7	4.9 ± 0.9
NP-E pH 6	427 ± 18	0.07 ± 0.03	−16 ± 11	15.4 ± 0.2	–
NP-E pH 6.5	355 ± 38	0.09 ± 0.05	−32 ± 7	15.7 ± 0.7	–
NP-E pH 7.5	234 ± 20	0.01 ± 0.01	−38 ± 7	10.6 ± 0.3	–
NP-E pH 8	190 ± 4	0.05 ± 0.00	−39 ± 9	6.2 ± 1.9	–

Table 2. Characterisation of NPs with different plasmid amounts prepared at a pH of 6.5 ($n = 3$).

NP formulation (µg)	Size (nm)	Polydispersity index	Zeta potential (mV)	Particle yield (mg)	Plasmid load (µg/mg)
0	356 ± 5	0.09 ± 0.05	−31 ± 7	16.1 ± 0.7	–
10	326 ± 4	0.03 ± 0.01	−38 ± 5	15.6 ± 0.8	0.7 ± 0.3
50	248 ± 5	0.04 ± 0.01	−42 ± 6	16.7 ± 1.1	2.7 ± 0.5
100	214 ± 10	0.04 ± 0.03	−43 ± 3	14.2 ± 1.6	5.8 ± 0.7
200	185 ± 10	0.05 ± 0.02	−42 ± 3	10.1 ± 1.7	16.5 ± 3
300	174 ± 10	0.04 ± 0.01	−43 ± 5	9.2 ± 1.1	21.2 ± 3.2

the therapeutical use is a sufficient plasmid release from the particles. On the one hand, the DNA is protected in the matrix of the NPs (Arnedo et al., 2004), on the other hand a high binding efficiency may negatively affect the release of the plasmids. In our storage experiments, no plasmids were detectable in the supernatants for 2 weeks (data not shown). This may be due to strong interactions of the polymer with the plasmids, which are

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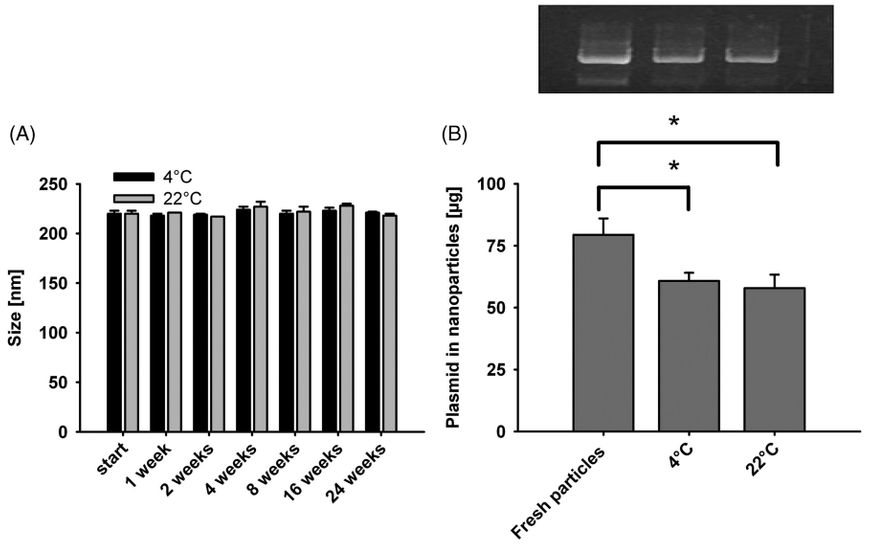

Figure 2. Size of NPs (A) and amount of incorporated plasmid (B) stored over 24 weeks at different temperatures. Representative agarose gel electrophoresis for entrapped plasmid is shown in the inset in B ($n = 3$; mean ± SD).

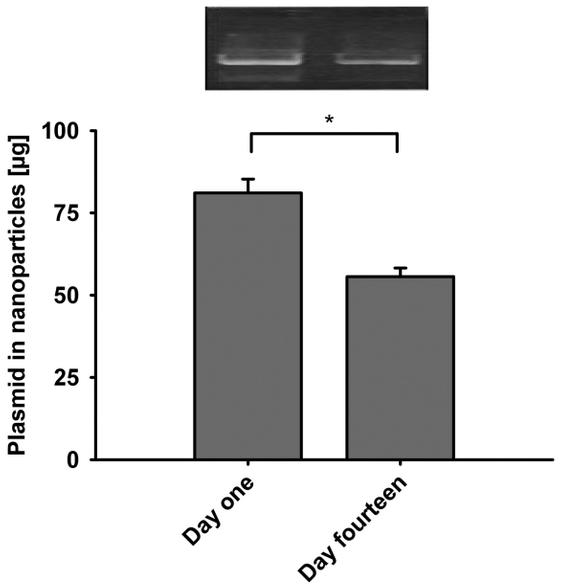

Figure 3. Amount of plasmid entrapped in NPs at days 1 and 14 of the release study. Representative agarose gel electrophoresis for entrapped plasmid is shown in the inset ($n = 3$; mean ± SD).

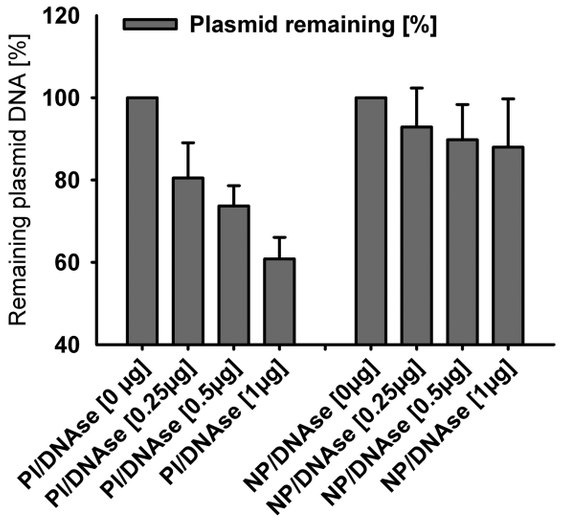

Figure 4. DNAse stability: naked DNA (left columns) was degraded in a DNAse 1 concentration-dependent manner whereas the NPs (right columns) protected the incorporated DNA from a degradation by DNAse 1 ($n = 3$; mean ± SD).

assumed to be higher at pH 6.5 near the isoelectric point of HSA at which the polymer lost most of his negative charge (Langer et al., 2003). In the release study at 36°C, the amount of plasmid inside NPs decreased significantly over the 2 weeks. This decrease was higher than in our stability test after 24 weeks storage at 4°C or at room temperature (Figure 3). However, in both cases more than 50% plasmid was still intact.

Protection against DNAse

To achieve an efficient delivery of the plasmid DNA by NPs, protection of the incorporated DNA is mandatory. Therefore, the stability of NP encapsulated plasmids as well as naked plasmids were investigated. After the treatment with DNAse 1, an enzyme concentration-dependent degradation of free plasmids was observed whereas plasmids entrapped in NPs appear to be stable (Figure 4). These results indicate that encapsulated plasmids were protected against degradation by DNAse 1. Hence, this stability problem can be solved by the employment of NPs.

Scanning electron microscope

The scanning electron microscope (SEM) pictures confirmed the results from PCS. Three exemplary pictures

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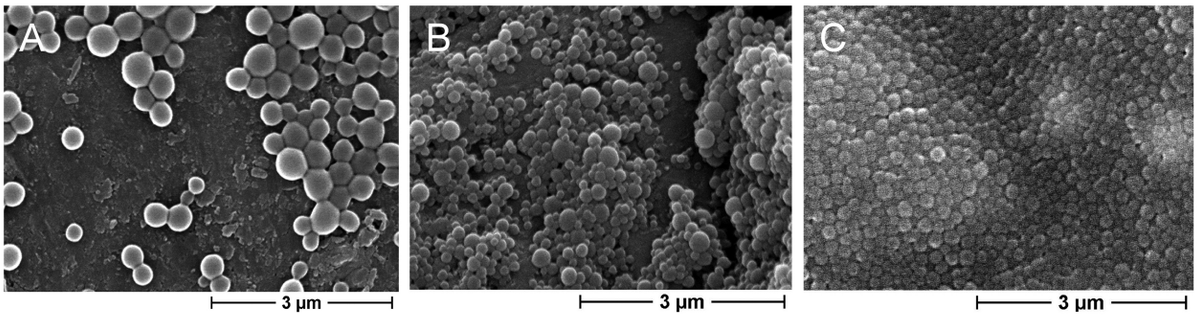

Figure 5. Exemplary SEM pictures taken from different NP preparations: (A) NPs without incorporated plasmids, (B) NPs with 100 µg incorporated plasmid, (C) NPs with 300 µg plasmid. A size-reducing effect of the incorporated drug is visible.

from NPs loaded with increasing plasmid concentrations are shown in Figure 5 with an obvious size reducing effect at higher plasmid concentrations. At a plasmid content of 300 µg not only very small particles but also a pretty narrow size distribution is visible indicating that all NPs probably have comparable amounts of plasmid entrapped. This interrelationship was further confirmed in plasmid quantification experiments. Results from these experiments underline this hypothesis, as at plasmid concentrations of 300 µg not all DNA was incorporated into the particles, leading to a homogeneous saturation of the albumin NPs with plasmid DNA. These pictures also indicate that NPs with encapsulated plasmids have a smaller diameter than NPs without incorporated DNA. One other important factor determining the size of albumin NPs is the pH value (Langer et al., 2003) due to its influence on the amount of negative surface charges in the molecule that in turn govern the repulsive forces. During preparation of NPs, the albumin molecules and the plasmid DNA precipitate together and form NPs (Steinhauser et al., 2009). As described above, negative charges reduce the agglomeration of the macromolecules because of resulting repulsive forces between the molecules. This effect leads to smaller particles during their formation. These charges are lower near the isoelectric point of albumin leading to larger micro- or macro-particles. In a mixture of albumin and plasmids, the negatively charged plasmids interact with the albumin molecules and inhibit the agglomeration and formation of larger particles.

Knockdown of survivin expression by miRNA HSA-NP

As shown in Figure 6, a clear correlation between the amount of incorporated survivin-miRNA plasmid and the reduction of the survivin protein expression was evident. At a concentration of 300 µg plasmid, the survivin expression is reduced to 50% of the protein level compared to controls. A significant reduction was further evident between 200 and 0 µg ($p = 0.013$) and between 300 and 0 µg ($p = 0.001$). In contrast, the scrambled plasmid loaded NPs exhibit no clear trend in the down regulation of survivin with no obvious reduction at the highest plasmid content of 300 µg.

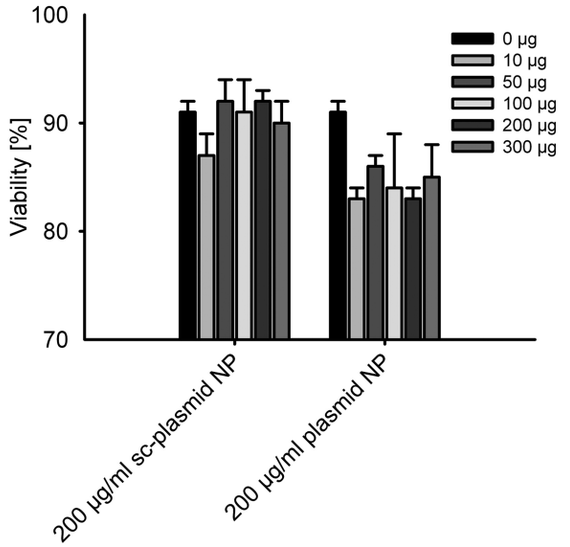

Figure 6. Toxicity of survivin-miRNA plasmids in NPs as well as scrambled plasmids (200 µg/mL). Only a slight difference is evident indicating that there is only a minor effect of the plasmids ($n = 3$; mean ± SD).

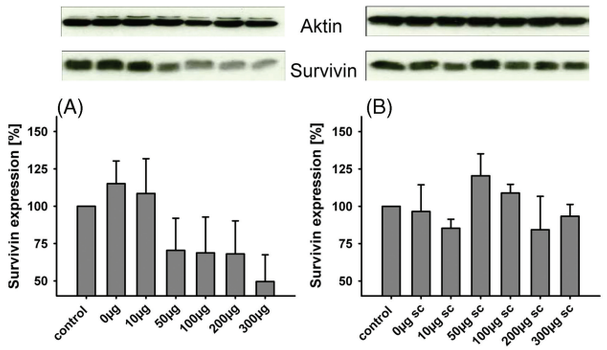

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Figure 7. Reduction of survivin expression with survivin-miRNA NP (A) as well as NPs with scrambled plasmids (B) a significant decrease between 300 mg survivin-miRNA plasmid and 300 mg sc scrambled plasmid ($p = 0.029$) is visible. In (A), a significant reduction between 0 and 200 mg ($p = 0.013$) and between 0 and 300 mg ($p = 0.001$) and 10 and 300 mg ($p = 0.007$) of survivin-miRNA plasmids is evident. Representative western blots show survivin level (bottom) and β-actin (top) for standardization ($n = 3$; mean ± SD).

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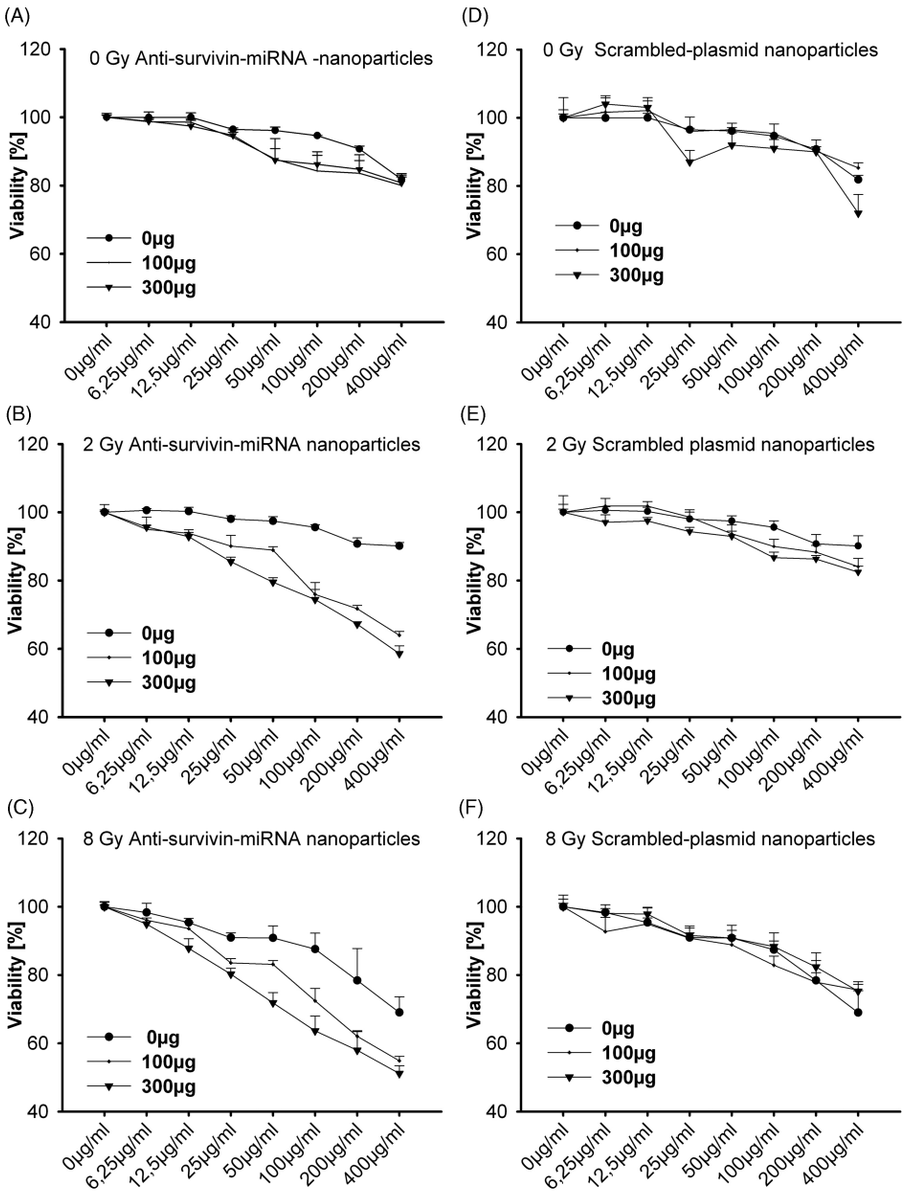

Figure 8. Viability of SW480 cells after treatment with different NPs in varying concentrations and radiation doses. A, B and C denote survivin miRNA plasmid NPs and D, E and F scrambled plasmid incorporated NPs ($n = 3$; mean ± SD).

Toxicity of survivin HSA-NP

Previous studies (Kratz, 2008; Wagner et al., 2009) indicated that HSA or HSA-derived NPs display no toxicity and are well tolerated in animal models. By contrast, toxicity may arise from incorporated plasmids by down regulation of survivin or by unspecific effects in the cells. Therefore, HSA-NP formulations were tested at different concentrations containing survivin miRNA and scrambled control plasmids.

The results illustrate a well tolerability of the NPs. A slight difference between the two formulations was observable (Figure 7), which indicated that there was a minor apoptosis inducing effect of the survivin miRNA without ionising radiation or any further treatment. An induction of apoptosis was also reported for survivin antisense (Lladser et al., 2011). In addition to the survivin effect, we observed a slight reduction in viability compared to untreated cells at the highest NP concentration in both cases, that may be due to unspecific effects on the cells.

Increased toxicity of HSA-NP if combined with ionising radiation

Accordingly, NP preparations with 0, 100 and 300 µg survivin-miRNA plasmids were irradiated with single doses ranging from 0 to 8 Gy and subjected to MTT assays. As displayed in Figure 8, just a light difference in cellular viability was observed in cells treated with scrambled or survivin miRNA carrying NPs in non-irradiated SW480 cells. Irradiation with a dose of 2 Gy

in the survivin miRNA treated cells decreased viability to 60% as compared to scrambled control or non-irradiated cells, where as a 8 Gy exposure resulted in a significant reduction of viability in all tests, again with the highest effect in survivin miRNA NP treated cells.

These results indicate the important role of survivin in cancer therapy. Cells without downregulated survivin status showed a decreased response to irradiation compared to cells with a partly reduced survivin expression. This effect has been reported in many previous studies *ex vivo* and *in vivo*, as well (Rödel et al., 2005). Furthermore, overexpression of survivin in cancer cells is associated with increased malignancy and a poor response to therapy (Capalbo et al., 2007).

Basic principle of most chemotherapeutics and ionising irradiation is the generation of DNA-damage and subsequent cell death by apoptosis. Survivin not only counteracts apoptosis but also promotes cell proliferation, regulates cell division and ensures cell survival and response to unfavourable surroundings (Altieri, 2008). Moreover, survivin is reported to be an inducible radiation resistance factor (Asanuma et al., 2000) and a therapeutic attenuation of the protein by siRNA, antisense-oligonucleotides or small molecule suppressors is shown to sensitise tumour cells towards irradiation (Rödel et al., 2011). The underlying molecular mechanisms of this sensitisation exceed a simple inhibition of apoptotic pathways and also involve caspase-independent mechanisms like regulation of the cell cycle and modulation of DNA-damage repair (Reichert et al., 2011). These characteristics in line with a prominent overexpression of survivin in cancerous tissue pinpoint the importance of the protein as a suitable target for a molecular based therapy of cancer.

Conclusion

In this proof-of-principle study, we show that it is possible to produce a stable and monodisperse HSA-based NP drug carrier system for survivin-specific miRNA constructs. Data from cell culture experiments suggest that this NP-delivery system could be used to down regulate the expression of survivin and as a consequence to increase the sensitivity of tumour cells, that are otherwise resistant to ionising radiation. Another positive property is the lack of toxicity of these NPs against non-radiated cells and their easy handling and fabrication.

These results suggested that survivin-miRNA NPs could be an efficient and safe gene carrier system for RNAi therapeutics in tumour therapy.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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9. Lebenslauf

Name:	Sebastian Reichert
Geburtsdatum:	08.03.1983
Geburtsort:	Saarlouis
Staatsangehörigkeit:	deutsch

Universitäre Ausbildung

seit 10/2009	Promotion am Klinikum der Johann Wolfgang Goethe-Universität in der Klinik für Strahlentherapie und Onkologie, im Labor für Molekulare Strahlenbiologie
2009	Abschluss der Biologiestudiums, Diplom
2008 – 2009	Diplomarbeit am Clemens-Schöpf-Institut für Biochemie an der TU-Darmstadt (Thema: Bakterielle Oberflächen-präsentation der Lipase LipA und ihrer Foldase LipH)
2003 – 2009	Studium der Biologie an der TU-Darmstadt

Zivildienst

2002 – 2003	Zivildienst im Altenheim Viktoria, Klein-Gerau

Schulische Ausbildung

2002	Allgemeine Hochschulreife
1993 – 2002	Prälat-Diehl-Gymnasium Groß-Gerau
1989 – 1993	Erich-Kästner-Schule Klein-Gerau

10. Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Sämtliche aus fremden Quellen direkt oder indirekt übernommene Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und noch nicht veröffentlicht.

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